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PROGNOSTIC BIOMARKERS IN COLORECTAL CANCER

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ACADEMIC DISSERTATION

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To my family

PROGNOSTIC BIOMARKERS IN COLORECTAL CANCER

CONTENTS

1. ABBREVIATIONS

ACPS	Australian Clinico-Pathological Staging
ACVR2	Activin Type II Receptor
AJCC	American Joint Committee on Cancer
APC	adenomatous polyposis coli
AFAP	attenuated familial adenomatous polyposis
BM	basement membrane
BMPR1A/ALK3	type I member of the TGF beta receptor superfamily of transmembrane ser/thr kinases
BRAF	v-Raf murine sarcoma viral oncogene homolog B1
CA19-9	carbohydrate antigen 19-9
CA 242	carbohydrate antigen 242
CEA	carcinoembyonic antigen
CIN	chromosome instability
CT	computed tomography
CIMP	CpG island methylator phenotype
CRC	colorectal cancer
DALM	dysplasia-associated lesion or mass
DNA	deoxyribonucleic acid
ECM	extracellular matrix
EGFR	epidermal growth factor receptor
EUS	endoscopic ultrasound
FAP	familial adenomatous polyposis
5-FU	5-fluorouracil
GTPase	guanosine triphosphatase
H&E	haematoxylin-eosin
HNPCC	hereditary nonpolyposis colorectal cancer
IBD	inflammatory bowel disease
KRAS	Kirsten ras
LKB1/STK11	liver kinase B1/ Serine/threonine kinase 11
PMS2	DNA mismatch repair gene homologue
RAF	receptor tyrosine kinase effector
RAS	ras-p21 protein coding gene
MAPK	mitogen-activated protein kinase
MLH1	methylation of MutL homologue 1
MLH3	mutL homolog 3 gene
MRI	magnetic resonance imaging
MSH2	inactivation of MutS homologue 2
MSH6	mutS homolog 6 gene
MSI	microsatellite instability
MSI-H	multiple allelies of varying length formation
MMP	matrix metalloproteinase
MMR	mismatch repair
MUTYH	mutY homolog gene
NSCLC	non-small cell lung cancer

PET/CT	positron emission tomography
PI3K	phosphoinositide 3-kinase
PJS	Peutz-Jeghers syndrome
PMS2	mismatch repair endonuclease
PROX1	Prospero homeobox protein 1
PSTI	pancreatic secretory trypsin inhibitor
18qLOH	loss of heterozygoty of chromosome 18
SPINK 1	serine protease inhibitor Kazal-type 1
TACSTD1	tumor-associated calcium signal transducer 1
TATI	tumour-associated trypsin inhibitor
TCF4	transcription factor 4
TEM	transanal endoscopic mucosectomy
TGF	transforming growth factor α
TGFBR1	transforming growth factor, beta receptor I
TGFBR2	transforming growth factor, beta receptor II
TIMP-1	tissue inhibitor of metalloproteinases-1
TILs	intratumoral lymphocytes
TMA	tissue microarray
TME	total mesorectal excision
TNM	tumor node metastasis
TP53	tumour protein p53 gene
UC	ulcerative colitis
UICC	Union Internationale Contre le Cancer
VEGF	vascular endothelial growth factor

2. ORIGINAL PUBLICATIONS

This thesis is based on the following publications, referred to in the text by their Roman numerals I-V.

I. Koskensalo S, Louhimo J, Nordling S, Hagström J, Haglund C. MMP-7 as a prognostic marker in colorectal cancer.
Tumour Biol 2011;32(2):259-64.

II. Böckelman C, Koskensalo S, Hagström J, Lundin M, Ristimäki A, Haglund C. CIP2A over-expression is associated with c-Myc expression in colorectal cancer.
Cancer Biol Ther 2012;13(5):289-95. *

III. Koskensalo S, Hagström J, Louhimo J, Stenman U-H, Haglund C. Tumour-associated trypsin inhibitor TATI is a prognostic marker in colorectal cancer.
Oncology 2012;82(4):234-241.

IV. Koskensalo S, Hagström J, Linder J, Lundin M, Sorsa T, Louhimo J, Haglund C.
Lack of MMP-9 expression is a marker for poor prognosis in Dukes´ B colorectal cancer.
BMC Clin Pathol. 2012;12(1):24:1-7.

V. Koskensalo S, Louhimo J, Hagström J, Lundin M, Stenman U-H, Haglund C. Concomitant tumor expression of EGFR and TATI/SPINK 1 associates with better prognosis in colorectal cancer.
PLOS ONE, in press.

*Publication II was also included in the thesis of Camilla Böckelman. Only its results on p53 and Ki-67 are included in this thesis.

3. ABSTRACT

Background and aims: The most important prognostic factor in colorectal cancer (CRC) is tumour stage. Prognosis of local tumours is good, but in tumours with lymph node or distant metastasis, the prognosis is worse. Patients with stage III (Dukes' C) tumours usually receive adjuvant chemotherapy. Patients with stage IV (Dukes' D) tumours cannot be treated curatively by surgery alone and usually receive chemotherapy. In stage II (Dukes' B) disease, adjuvant chemotherapy is recommended for patients at risk for recurrence, such as with tumours with vascular or perineural invasion, or in cases with emergency surgery or insufficient lymph-node harvest. In order to identify better those patients requires additional prognostic factors like biomarkers.

Material and methods: Clinical data came from 643 consecutive patients who underwent surgery for colorectal cancer at the Department of Surgery, Meilahti Hospital, Helsinki University Central Hospital, between 1982 and 1998. Clinical data and archival tissue specimens were available from 623 cases. For MMP-9, a validation series of 213 patients treated between 1998 and 2001 was included. Survival data came from the Population Register Centre of Finland and Statistics Finland. Tissue microarray (TMA) blocks were prepared from re-evaluated histological archive blocks. TMA slides were stained with MMP-2, MMP-7, MMP-8, MMP-9, TATI, trypsinogen-1, trypsinogen-2, p53, Ki-67, and EGFR antibodies. Correlation of immunoexpression of markers with clinicopathological variables was assessed. Survival analysis was performed by the Kaplan-Meier method, and multivariate Cox proportional hazards model.

Results: Study I showed strong MMP-7 to be an independent prognostic marker for 5-year survival, but later the difference faded. In Study II, no association was observable between p53 or Ki-67 expression and survival. In Study III, TATI immunoexpression was an independent prognostic marker for improved survival, particularly in subgroups of trypsinogen-1- and trypsinogen-2-positive patients, although trypsinogen-1 and trypsinogen-2 were not prognostic factors. In Study IV, MMP-9 expression was an independent prognostic marker of favourable survival in Dukes' B patients, but the validation series did not confirm these results. MMP-2 and MMP-8 immunoexpression lacked any correlation with prognosis. In Study V, EGFR+TATI+ patients had significantly better prognosis than did those with EGFR+TATI-, EGFR-TATI+, or EGFR-TATI-.

Conclusion: MMP-7, MMP-9, TATI, and the TATI-EGFR combination can all serve as prognostic biomarkers in CRC.

4. INTRODUCTION

The incidence of colorectal cancer in industrialized countries is increasing. Although a genetic predisposition exists, almost 95% of cases are sporadic (Cunningham et al. 2010). The development of cancer is a multistep process involving different phenomena like inflammation, genetic instability, and gene mutations. Most colorectal carcinomas develop by what is called the adenoma-carcinoma sequence (Fearon and Vogelstein 1990).

The risk of death from colorectal cancer depends mainly on stage of the disease at diagnosis. In Finland in 2011, cumulative 5-year survival with colon cancer was 60% in male and 61% in female patients, and for rectal and anal cancer, 62% and 65% (Finnish Cancer Registry www.cancer.fi). Patients with local tumours have the most favourable prognosis. Patients with stage III (Dukes' C) tumours usually receive andjuvant chemotherapy. Patients with stage IV (Dukes' D) tumours cannot be treated curatively by surgery alone, and they usually receive chemotherapy. In stage II (Dukes' B) disease, about 15 to 20% of patients develop recurrent disease, and patients with known risk factors such as vascular or perineural invasion, or in cases requiring emergency surgery, adjuvant chemotherapy is recommended. To better identify patients at risk requires additional prognostic factors.

Matrix metalloproteinases (MMPs) and trypsin are enzymes capable of degrading extracellular matrix and basement membranes, a requirement for local tumour spread and metastasis. In addition to its proteolytic activity, trypsin is able to activate MMPs. A trypsin inhibitor called tumour-associated trypsin inhibitor (TATI) is expressed together with trypsin in many cancer types. TATI is also a ligand of epidermal growth factor receptor (EGFR), which plays a role in colorectal carcinogenesis. Mutation in tumour suppressor gene p53 leads to disturbances in apoptosis, in angiogenesis, cell cycle, and in genomic maintenance; mutation of p53 occurs in many cancers. Ki67 is an antigen associating with cell proliferation; it is expressed in all phases of the cell cycle except G0.

The aim of this study was to evaluate the expression of MMP-2, MMP-7, MMP-8, MMP-9, trypsinogen-1 and trypsinogen-2, TATI, EGFR, p53, and Ki-67 in colorectal cancer and to evaluate their association with patient prognosis in colorectal cancer.

5. REVIEW OF THE LITERATURE

5.1. Epidemiology and incidence

Colorectal cancer (CRC) is one of the most common malignancies worldwide; with about 1.23 million new cases registered in 2008; it is the fourth most common cancer in men and the third in women. CRC incidence varies by geographical area, high-risk areas being industrialized, high economic-level areas such as North America, northern and western Europe, Australia, New Zealand, and Japan (Boyle et al. 2008, Ferlay et al. 2010). In Finland, the CRC incidence is increasing, with approximately 2800 new cases diagnosed in 2011; the age-adjusted incidence for males was 27.9/100 000 and for females19.5/100 000. CRC is the third most common malignancy in Finland; in females only breast cancer, and in males, prostate and lung cancer have higher incidences. Two-thirds of CRC tumours occur in the colon, and one-third in the rectum (Finnish Cancer Registry). The majority of colorectal cancer cases are sporadic; of all cases, genetic carcinoma syndromes account for less than 6% (Aaltonen et al. 2007).

The mortality rate of CRC depends on the availability of appropriate treatment, being lower in high-risk areas (Boyle et al. 2008, Ferlay et al. 2010). In Finland, the mortality rate for colon cancer is approximately 10.3/100 000, and for rectosigmoid and rectal cancer 7.1/100 000. In Finland in 2011, about 1700 individuals died from CRC. Cumulative 5-year survival of colon cancer was 60% for male and 61% for female patients, and of rectal cancer, 62% and 65% (Finnish Cancer Registry).

5.2. Molecular and genetic pathobiology

The development of cancer is a multistep process including different phenomena in normal cells leading to malignant development and progression. Colorectal cancer is derived from accumulation of sequential alterations in tumour suppressor and DNA repair genes and proto-oncogenes (Arnold et al. 2005). Most colorectal carcinomas develop by what is called the adenoma-carcinoma sequence (Fearon and Vogelstein 1990) (Figure 1). According to the first described model, tumours develop by transformation of normal epithelium into benign neoplasia, then tubular or tubulovillous adenoma, and further into carcinoma (Fearon and Vogelstein 1990). Later, also serrated adenomas and transitional serrate adenomas have revealed their malignant transformation capacity (Jass et al. 2004, Goldstein et al. 2006). Their genetic pedigree differs; serrated polyps are associated with microsatellite instability and aberrant DNA methylation at CpG islands, whereas tubular adenomas arise by APC gene inactivation (Noffsinger et al. 2009).

The most common genomic instability in colorectal cancer, found in 85% of tumours, is chromosome instability (CIN) (Grady et al. 2004). CIN alterations can be chromosomal amplifications and translocations or allelic losses. More than 90% of tumours have mutations in the APC gene, and 50% in the KRAS gene. The allelic loss of 18q is found in 80% of tumours and TP53 mutations in 70% (Seth et al. 2009). Other mutations occur more rarely.

Microsatellite instability (MSI) is observable in 15% of sporadic tumours and in almost all Lynch syndrome cases (Chan et al. 2005, Hamelin et al. 2008). In these tumours, the mismatch-repair is non-functional, leading to multiple alleles of varying length formation, called MSI-H (Imai et al. 2008, Bellizzi et al. 2009). In sporadic cases, the predominant error is MLH1 gene-promoter methylation. Lynch syndrome includes germline mutations in the mismatch repair genes (MSH2, MLH1, MSH6, or PMS2) or in the regulatory gene (TACSTD1) (Ligtenberg et al. 2009).

CpG island methylator phenotype (CIMP) tumours have methylated promoter regions. Due to methylation, chromosomal structures are changed, causing inhibition of gene expression (Curtin et al. 2011). Methylation of apoptosis-promoting genes leads to decreased apoptosis (Sandmeier et al. 2009). Carcinomas with CIMP often have MSI-H, due to the methylation of MLH-1. CIMP tumours with BRAF and MSI-H mutations are called CIMP1, and tumours with KRAS mutation without MSI, CIMP2 (Shen et al. 2007, Suehiro et al. 2008).

5.2.1. Specific genetic alterations and signal pathways

The WNT-β-catenin signalling pathway is affected by APC gene mutations found in FAP and in 70% of sporadic CRCs (Morin et al. 1997, Chung et al. 2000). Normally the APC protein inhibits WNT signalling by targeting β-catenin for ubiquitin-mediated proteasomal degradation. The mutation leads to increased WNT signalling and promotion of β-catenin/TCF4-mediated transcription (Caldwell et al. 2004).

The transcription factor PROX1 is a target of the β-catenin/TCF signalling pathway and is overexpressed in CRCs. Gene activation promotes tumour growth and malignant transformation (Petrova et al. 2008, Skog et al. 2011).

TGF-β signalling is a tumour-suppressor pathway, impairment of which occurs in the majority of CRCs (Chittenden et al. 2008). TGF-β signalling-associated mutations are observable in TGFBR2, TGFBR1, SMAD2, SMAD4, and ACVR2 genes (Eppert et al. 1996, Deacu et al. 2004, Grady et al. 2004), and lead to spontaneous proliferation of cells (Xiong et al. 2002). The TGF-β pathway is also disturbed in genotype 18qLOH (loss of heterozygosity of chromosome 18) (Fearon and Vogelstein 1990).

KRAS is a proto-oncogene of the EGFR-mediated signal pathway, and its activation leads to activation of the mitogen-activated protein kinase (MAPK). Activating mutations lead to proliferatory stimulus. KRAS mutations occur in about 40% of CRC cases (Downward et al. 2003). BRAF is the second molecule of the EGFR-mediated signal pathway. BRAF-gene mutations, found in 10 to15% of CRCs (Siena et al. 2009), are specific for carcinomas originating from serrate adenomas (O´Brien et al. 2006).

The PI3K pathway is another downstream pathway of EGFR signalling. Mutations of PI3K-associated genes occur in 40% of CRCs (Parsons et al. 2005). These mutations can promote transformation of adenomas into carcinomas (Samuels et al. 2004). Other mutations able to promote malignant transformation of adenomas are TP53 tumour-suppressor gene mutations (Vogelstein 2000). The p53 protein plays a role in signalling of cell-cycle arrest and apoptosis (Steele et al. 2005).

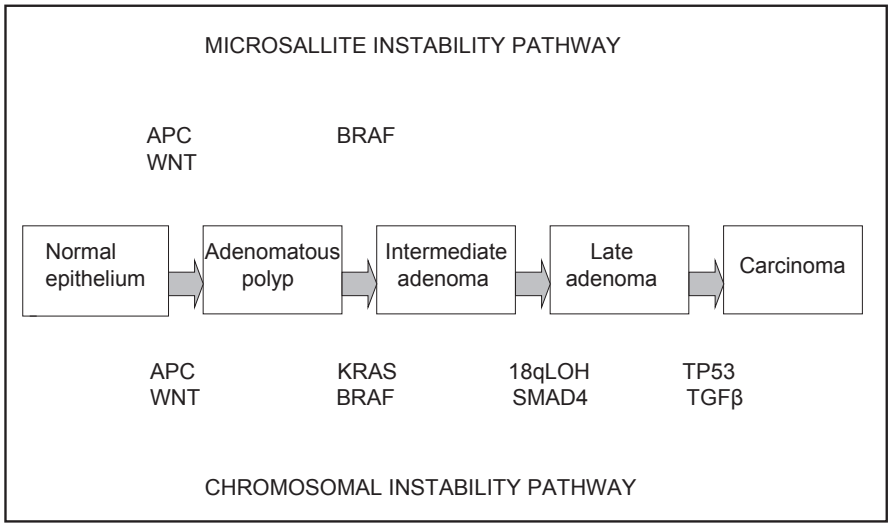

Figure 1. Pathways of colorectal cancer progression: APC gene mutation initiates early adenomatous formation. Serrated adenoma pathway with BRAF mutations leads to sporadic MSI tumours. Cancer progression via KRAS and 18qLOH mutations occurs in the CIN pathway. (Modified from Fearon and Vogelstein´s model, 1990)

5.3. Risk factors

5.3.1. Dietary factors and life-style

A "Western type" diet with high caloric, meat, and animal-fat intake, and with low fruit, vegetable, and fibre intake is a risk factor for CRC (Key et al. 2011). Smoking is known to elevate the risk both for adenomatous polyps and for colorectal cancer (Slattery et al. 2004, Botteri et al. 2008). Obesity associates with an elevated risk for CRC (Moghaddam et al. 2007, Donohoe et al. 2010). An inverse association exists between CRC incidence and D-vitamin and calcium intake (Kallay et al. 2005). Alcohol is one of the known risk factors (Giovannucci et al. 2004). On the other hand, coffee consumption might play a protective role against CRC (Sinha et al. 2012). Nonsteroidal anti-inflammatory drugs (Cuzick et al. 2009) and oestrogen replacement are also known to be preventive factors (Barone et al. 2012).

5.3.2. Hereditary syndromes

5.3.2.1. Lynch syndrome

Lynch syndrome, previously known as hereditary nonpolyposis colorectal cancer (HNPCC), is an autosomal dominant disease with a high lifetime risk for developing into colorectal cancer (Calvert et al. 2002). Mutations in this syndrome occur in DNA mismatch repair (MMR) genes: most are in MLH1 and MSH2 genes, but also in MLH3, MSH6, and PMS2 (Sankila et al. 1996, Lynch et al. 1999, Peltomäki et al. 2004, Woods et al. 2007). In half of all HNPCC families, neither MMR mutations nor microsatellite instability is evident, in which case it is not called Lynch syndrome (Vasen et al. 2007).

About 2 to 3% of all colorectal cancers are HNPCC (Harford et al. 2006). Typically, patients are younger than in sporadic cases (mean 40-50 years), and synchronous or metachronous cancers are

present in 18% of cases (Vasen et al. 2005). The prognosis of patients with tumours caused by the MMR gene mutation is better than that of sporadic cases (Aarnio et al. 1998). Because of the high risk of developing malignancy, screening with regular colonoscopies, and extended colectomy if cancer occurs is recommended (Vasen et al. 2013).

5.3.2.2. FAP

Familial adenomatous polyposis (FAP) is an autosomal dominant syndrome caused by mutations in the adenomatous polyposis coli (APC) gene (Hamilton et al. 1992, Narayan et al. 2003, Burt et al. 2005). Among colorectal cancers, it accounts for about 1%. Typically the patients have hundreds or even thousands of adenomatous polyps in the colon and rectum. The lifetime risk for colorectal cancer is 100% , with the mean age of diagnosis of cancer at about 40 (Galiatsatos et al. 2006). Prophylactic colectomy or proctocolectomy is therefore usually performed. Extracolonic lesions like gastric, duodenal, and small bowel adenomatous polyps also often occur (Bosman et al. 2010). In attenuated familial adenomatous polyposis (AFAP), the number of polyps is less than in FAP, and the risk for cancer and extracolonic manifestations is lower (Burt et al. 2004).

5.3.2.3. Other syndromes

Other rare genetic syndromes with risk for colorectal cancer are MUTYH-associated polyposis (MAP) (previously called MYH-associated polyposis) (Sieber et al. 2003), juvenile polyposis (Järvinen et al. 1984) and Peutz-Jeghers syndrome (Jeghers et al. 1949).

5.3.3. Inflammatory bowel disease (IBD)

Chronic inflammatory bowel disease (IBD), especially ulcerative colitis (UC), is a risk factor for CRC (Bernstein et al.2001), with risk increasing by duration of disease (Eaden et al. 2001). Unlike in other sporadic carcinomas, which typically develop via the adenoma-carcinoma sequence, a precancerous lesion in IBD can be flat, with a normal endoscopic appearance (Rhodes et al. 2002) or can be a dysplasia-associated lesion or mass (DALM) (Odze et al. 1999). Increased resistance to apoptosis and increased secretion of proinflammatory cytokines such as interleukin-6 lead to chronic activation of the mucosal immunosystem (Mudter et al. 2007). Proinflammatory cytokines can promote cancer development in UC (Atreya et al. 2008), and risk factors for cancer are UC duration longer than 10 years, pancolitis, and left-sided disease (Ullman et al. 2009). The prognosis of UC-associated cancer is worse than that of sporadic cases (Aarnio et al. 1998).

5.4. Diagnosis

5.4.1. Symptoms and signs

Many colorectal cancer patients lack disease symptoms. Others suffer from hematochezia or anaemia and altered bowel habits such as diarrhoea or constipation. Especially with rectal tumours, mucous and bloody stools and urgency may occur. Fatigue, weight loss, abdominal distension or pain, and bowel

obstruction and perforation are symptoms of advanced disease, as are symptoms caused by metastatic disease, such as liver enlargement or ascites (Hamilton et al. 2004).

5.4.2. Clinical examination

Over 50% of rectal cancer cases can be found by digital rectal examination (Lepistö et al. 2009). This is also an important method to evaluate tumour fixation into pelvic structures and to notice blood in stools. A large colonic tumour can be found by abdominal palpation.

5.4.3. Endoscopy

The most important diagnostic tool for CRC is colonoscopy. It allows evaluation of tumour size and location, and allows biopsies for histology and tattooing of the tumour. In rectal cancer, rigid rectoscopy may allow evaluation of the distance from the lowest part of the tumour to the anal canal, enabling a choice of appropriate surgical technique.

5.4.4. Preoperative radiological staging

Preoperative thoracic and abdominal computed tomography (CT) allows detection or exclusion of metastatic disease, and it may help in evaluating the location and size of the tumour, its relation to adjacent organs, and even its invasion depth. These variables are important for preoperative staging and for determining the proper surgical technique. The sensitivity of CT for lymph node metastasis (N stage) is 76%, and its specificity 55%; in evaluation of distant metastasis (M stage), sensitivity is 85% and specificity 98% (Leufkens et al. 2011).

In rectal cancer, CT is used for evaluating distant metastases, whereas magnetic resonance imaging (MRI) is the examination method of choice for local staging. It enables evaluation of the distance of the tumour from the anal canal, depth of invasion, and enlargement of lymph nodes, all of which influence the choice of preoperative treatment and operative technique. MRI is sensitive in evaluating tumour invasion depth. The MERCURY group study (2007) showed MRI to be equal to histopathological evaluation of extramural invasion depth. In lymph-node evaluation, 85% accordance with histopathology can be reached by evaluating the irregularity of the nodal border and mixed intranodular signals (Brown et al. 2003), but micrometastases cannot be excluded (Dworak et al. 1989).

Intraluminal endoscopic ultrasound (EUS) can serve for local staging of rectal cancer, but only for flat and distal tumours. In determination of T stage, accuracy has been reported at 90% (Massari et al. 1998). EUS can find lymph nodes larger than 5 mm. EUS is, however, a very much operator-dependent examination.

Positron emission tomography (PET/CT) visualizes metabolic changes in cancer cells, but its sensitivity is poor (Heriot et al. 2004). It can be applied for detecting occult metastases, but it is unsuitable for preoperative local staging. Positron emission tomography together with MRI, a new alternative in evaluation of the local tumour staging, is under examination (Lambrecht et al. 2010).

5.5. Screening

Because CRC prognosis depends on disease stage, it is important to find the cancer as early as possible. Endoscopy or computed tomographic colonography can serve in screening, but each is expensive and resource-consuming. Occult blood test screening together with full colonoscopy in case of positive results is a means of finding symptomless tumours (Duffy et al. 2010). Pilot testing of screening based on faecal occult blood testing started in Finland in 2007 for the age cohort 60 to 69 years (Malila et al. 2011). Advantages of occult blood testing are non-invasiveness and inexpensiveness, but disadvantages are low sensitivity and specificity. More sensitive faecal testing than with occult blood is based on investigation of mutant DNA excreted from neoplastic lesions (Duffy et al. 2010). These tests are promising, but still very expensive and unsuitable for routine use.

5.6. Management

Management of colorectal cancer should be performed as multidisciplinary teamwork to improve tumour control and patient prognosis (Levine et al. 2012).

5.6.1. Surgery and preoperative treatment

5.6.1.1. Colon cancer

Standard operative techniques are based on location of the colonic cancer: right hemicolectomy for tumours of the caecum and ascending colon, extended right hemicolectomy for tumours located in the hepatic flexure or right side of the transverse colon, extended left hemicolectomy for tumours of the left side of the transverse colon or for flexura lienalis tumours, and left hemicolectomy for descending-colon or sigmoid tumours. At least a 5-cm margin on either side of the tumour is recommended. En-bloc excision of the mesocolon with proximal ligation of vessels is performed to improve the radicality of the operation in locally advanced disease and for staging. Tumours that invade adjacent organs or the abdominal wall, require en-block removal with healthy tissue margins. For small and local tumours, endoscopic resection is sometimes possible (Manfredi et al. 2006). Total mesocolon excision in right-sided colonic cancer may increase radicality (Eiholm et al. 2010).

Laparoscopy-assisted colorectal surgery has advantages over conventional techniques: shorter hospital stay, less postoperative pain, and better cosmetic results (Cera et al. 2005, Hotta et al. 2011). The oncological results are reported to be identical (Patankar et al. 2003, Reza et al. 2006), and an even better outcome has been reported for laparoscopically treated colon cancer in the first studies (Lacy et al. 2002).

5.6.1.2. Rectal cancer

For rectal cancer, resection of the rectum with total mesorectal excision (TME), is the method of choice (Heald et al. 1982). In TME, the rectum and mesorectum with lymphatics and venous drainage are resected within the mesorectal fascia, with proximal vessel ligation. For low- and mid-rectal tumours, a

distal margin of 1 to 2 cm is considered acceptable, and a transient ileo- or colostomy is recommended because of high risk for anastomotic leakage (Matthiessen et al. 2007). For high rectal cancers, partial excision of the mesorectum with an at least 5-cm distal margin is considered acceptable. If sparing of the anal sphincter is impossible, abdominoperineal excision with en-bloc removal of the rectosigmoid, rectum with TME, anal and perianal tissue, is necessary. The TME technique lowers the risk for local recurrence (Heald et al. 1998, Martling et al. 2000).

Preoperative radiation is recommended for T3-4 rectal tumours and for cases with suspected lymph-node metastases: a short course for T3 or lymph node-positive tumours or both, and a long course for T4 or fixed tumours (Schmoll et al. 2012). Short-course preoperative radiation of 25 Gy in 5-Gy fractions is given for 5 consecutive days. Long-course preoperative radiation takes 5 to 6 weeks with a total dose of 50.4 Gy combined with 5-FU- or capecitabine-based chemotherapy. The operation usually takes place 6 to 8 weeks after termination of radiation (Glimelius et al. 2008), but the recommended time-period between radiation and surgery may become longer in future (Stockholm III trial NCT00904813). Preoperative radiotherapy has been reported to improve survival (Colorectal Cancer Collaborative Group 2001, vanGijn et al. 2011) and to reduce local recurrences (Kapitejin et al. 2001). Compared to TME alone, preoperative radiotherapy plus TME reduces local recurrence by 50% (Peeters et al. 2007). Chemoradiotherapy can downstage the tumours in 60% of cases (Garcia-Aguilar et al. 2003) and make them resectable.

Some centres use laparoscopy-assisted surgery in rectal cancer treatment; it has similar benefits to laparoscopic colon cancer treatment, with the oncological results identical to those of open surgery (Arezzo et al. 2012, Green et al. 2013). Small rectal tumours may even be removable by colonoscopic or by transanal endoscopic mucosectomy (TEM).

5.6.2. Adjuvant treatment

The aim of adjuvant treatment is to reduce risk for recurrence and improve prognosis (Schmoll et al. 2012). Adjuvant treatment is based on 5-fluorourasil (5-FU) or cabecitabine, and together with oxaliplatin, it improves by 15 to 20% the prognosis in stage III CRC (Andre et al. 2004). Improvement also occurs in stage II tumours, but since here the overall prognosis is better, the final influence of 5-FU-based treatment on survival is only 3 to 5% (Gray et al. 2007). Due to side-effects, adjuvant treatment is recommended in stage II only for patients with risk factors such as emergency surgery, perforation, T4 tumour, less than 12 examined lymph nodes, vein or nerve invasion, or high-grade tumour (Schmoll et al. 2012). Cabecitabine, the predrug of 5-FU, has replaced 5-FU because of its better tolerance and resource advantages.

5.6.3. Treatment of metastatic colorectal cancer

CRC metastases can be operated on curatively, depending on size and location. Typical metastatic sites are liver, lungs, peritoneum, and other colonic segments (AJCC Cancer Staging Handbook, 2010). Of patients with liver metastasis at the time of diagnosis, up to 10 to 20% can be resected, and 10 to 15% of unresectable metastases can be resected after oncological treatment (Isoniemi et al. 2011). In Helsinki, the primary colorectal tumour is resected first, and if metastases respond to oncological treatment, liver or pulmonary surgery is performed later. After complete resection of hepatic metastases, 5-year survival can be up to 50% (Kopetz et al. 2009, Isoniemi et al. 2011). In stage IV CRC, or recurrent disease,

FU/cabecitabine, irinotecan, and oxaliplatin routinely serve as chemotherapy, usually combined with anti-EGFR-antibodies (cetuximab or panitumumab) for wt (wild type) KRAS tumours, or anti-VEGF antibody bevacizumab for KRAS-mutant patients (Gruenberger et al. 2008, Folprecht et al. 2010, Van Cutsem et al. 2011, Scmoll et al. 2012).

5.6.4. Palliative surgical treatment

In some cases, surgical resection of the primary tumour is not recommended, due to multiple metastases or due to invasion of adjacent organs, or because of the poor performance status of the patient. An obstructive tumour can be treated by a decompressing stoma or by-pass surgery, or by stenting the tumour.

5.7. Clinico-Pathological prognostic factors

5.7.1. Stage

The strongest prognostic factor in colorectal cancer is tumour stage (de Leon et al. 1987, Chapuis et al. 2011). In 1932, Dukes presented a staging system for rectal cancer including three stages: A, B, and C, with stage D later added by Turnbull et al. (1967). In Dukes' stage A, the tumour invades the submucosa or at most the muscularis propria. In stage B, the tumour invades through the bowel wall into the pericolic or perirectal fat. In stage C, the tumour invades or penetrates the bowel wall, and there occurs regional lymph node metastasis. In Turnbull´s modification, Dukes' stage D means that tumours have been resected nonradically, i.e. there are distant metastasis, or local radicality is insufficient (Turnbull et al. 1967). In 1982, a classification by the Australian Clinico-Pathological Staging (ACPS) was introduced (Davis and Newland 1982). Dukes' stage D was defined as clinical or microscopic evidence of remaining cancer tissue.

Dukes' stage is a good, but old-fashioned prognostic tool for evaluating prognosis in colorectal cancer. In 1982-1998, at Helsinki University Central Hospital, 5-year survival rates were 90% for Dukes' stage A, 75% for B, 50% for C, and below 10% for Dukes' D (Carpelan-Holmström et al. 1996, Louhimo et al. 2003).

TNM stage
The tumour node metastasis (TNM) staging system was first published in 1950 by the Union Internationale Contre le Cancer (UICC) (Denoix 1950). For colorectal cancer, this describes tumour infiltration (T), and prevalence of lymph-node (N) and of distant metastasis (M). The American Joint Committee on Cancer (AJCC) included prognostic TNM subgroups in their staging system (AJCC Cancer Staging Handbook. 1959). Later the AJCC and UICC were integrated, and the latest, the 7th edition, of the TNM classification was published in 2010 (Edge et al. 2010).

Table 1. Colorectal cancer staging: TNM and Dukes' 5-year survival by stage based on AJCC 2010.

Dukes	Stage	T	N	M	5-year survival (%)
	Stage 0	Tis	N0	M0	
Dukes' A	Stage I	T1,T2	N0	M0	93.2
Dukes' B	Stage II	T3,T4	N0	M0	
Dukes' B	Stage IIA	T3	N0	M0	84.7
Dukes' B	Stage IIB	T4a	N0	M0	72.2
Dukes' B	Stage IIC	T4b	N0	M0	
Dukes' C	Stage III	Any T	N1,N2	M0	
Dukes' C	Stage IIIA	T1,T2	N1	M0	83.4
		T1	N2a	M0	
Dukes' C	Stage IIIB	T3,T4a	N1	M0	64.1
		T2,T3	N2a	M0	
		T1,T2	N2b	M0	
Dukes' C	Stage IIIC	T4a	N2a	M0	44.3
		T3,T4a	N2b	M0	
		T4b	N1,N2	M0	
Dukes' D	Stage IVA	Any T	Any N	M1a	8.1
Dukes' D	Stage IVB	Any T	Any N	M1b	8.1

Table 2. TNM stages by UICC, 7th edition, 2010.

Primary Tumor (T)	
TX	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	Carcinoma in situ: intraepithelial or invasion of lamina propria
T1	Tumor invades submucosa
T2	Tumor invades muscularis propria
T3	Tumor invades through the muscularis propria into the subserosa, or into non-peritonealised pericolic or perirectal tissues
T4a	penetrates the surface of the visceral peritoneum
T4b	directly invades or is adherent to other organs or structures
Metastasis in 1 to 3 regional lymph nodes (N)	
N1a	Metastasis in one regional lymph node
N1b	Metastasis in 2-3 regional lymph nodes
N1c	Tumor deposit(s)
N2	Metastasis in four or more regional lymph nodes
N2a	Metastasis in 4-6 regional lymph nodes
N2b	Metastasis in seven or more regional lymph nodes
Distant Metastasis (M)	
M0	Not applicable metastasis
M1	Distant metastasis
M1a	Metastasis confined to one organ or site
M1b	Metastasis in more than one organ/site or the peritoneum

The original TNM stage is based on preoperative data. Normally, clinicians use the pTNM, also including data from the pathology report. TNM stage is a prognostic tool, but particularly a tool for planning surgical and oncological treatment.

5.7.2. Histological grade

Tumour histology is graded according to glandular formation. The WHO classification divides tumours according to histological differentiation into four groups: well-differentiated tumours (grade 1), moderately differentiated (grade 2), poorly differentiated (grade 3), and undifferentiated (grade 4) (Hamilton et al. 2000). High grade is an independent marker for poor prognosis (Compton et al. 2003, 2006).

5.7.3. Histological type

About 90% of CRCs are adenocarcinomas (Boyle et al. 2008), which are divided into subtypes. Mucinous adenocarcinomas are tumours with more than 50% of the lesion consisting of extracellular mucin. In signet-ring-cell carcinomas, over half of the cells show intracytoplasmic mucin, and the cells look like a signet ring. Rare CRC cancer types are medullary carcinoma, cribriform comedotype adenocarcinoma, small-cell carcinoma, micropapillary adenocarcinoma, and adenosquamous, spindle-cell, and undifferentiated carcinoma (Bosman et al. 2010).
Signet-ring cell, small-cell, and undifferentiated carcinomas are graded as high. The two first are independent markers for poor prognosis (Bernick et al. 2004, Kang et al. 2005).

5.7.4. Vascular, lymphatic, and perineural invasion

Vascular invasion is an independent marker of poor prognosis (Compton et al. 2006). Cancer cell invasion into extramural veins elevates risk for hepatic metastasis and adverse outcome (Blenkinsopp et al. 1981). Lymphatic invasion (Di Fabio et al. 2004, Maughan et al. 2007) and perineural invasion (Ueno et al. 2001, Fujita et al. 2007) are both independent markers of poor prognosis.

5.7.5. Tumour immunity

Tumour-related immune response is a prognostic factor; intratumoral lymphocytes (TILs) associate with survival and inversely with tumour stage (Ropponen et al. 1997). TILs also associate with absence of tumour budding (Zlobec et al. 2007). On the other hand, deficiency in peritumoral inflammatory reaction associates with poor prognosis (Losi et al. 2006). Peritumoral CD68+ macrophages associate with improved survival (Ålgars et al. 2011).

5.7.6. Tumour location

In older materials comparing colonic and rectal cancer, tumour location in the rectum was associated with poor prognosis (Park et al. 1999). Recently, differences have diminished due to improved adjuvant treatment and the surgical technique for rectal cancer, and in Finland as well as in Sweden, 5-year survival is similar for colonic and rectal cancer or even better in rectal cancer (Finnish Cancer Registry; Birgisson et al. 2005).

5.7.7. Mesorectal envelope and margins

Use of the TME technique in rectal cancer reduces local recurrences and improves survival (Heald et al. 1986, Arbman et al. 1996). Macroscopic evaluation of the rectal cancer preparate, i.e. completeness of the mesorectum, makes it possible to evaluate the prognosis (Kapiteijn et al. 2002). Microscopy allows circumferential as well as proximal and distal marginals to be evaluated, and these give prognostic data regarding local recurrence (Washington et al. 2009). If the circumferential margin is positive, the risk for recurrence is 3.5-fold and the risk of death 2-fold (Birbeck et al. 2006). In rectal tumours, a distal margin of 2 cm is adequate; in T1-2 tumours even 1 cm is sufficient (Washington et al. 2009). The lateral margins can be useful in prediction of local recurrence, metastasis, and survival (Nagtegaal et al. 2008). Similar results have been reported for improving survival in colon cancer by use of total mesocolon excision (Eiholm et al. 2010).

5.7.8. Perforation

Perforation caused by an obstructive tumour is associated with poor prognosis (Anwar et al. 2006).

5.8. Biomarkers

Development of cancer is a multistep process including different phenomena in the normal cell leading to malignant progression. A malignant tumour has to sustain proliferation signalling, evade growth-suppressors, resist cell death, induce angiogenesis, enable replicative immortality, and enable tumour invasion and metastasis (Hanahan and Weinberg 2000, 2011). Biomarkers or tumour markers are molecules, produced by cancer tissue or by normal tissue in response to a malignant tumour; they reflect these features of malignancies. Biomarkers, detectable in tissues or secreted into body fluids, can serve in screening for and diagnosis of cancer, but also in evaluation of prognosis and for monitoring of patients already treated or now under treatment.

5.8.1. Serum tumour markers

Carcinoembryonic antigen (CEA) is a glycoprotein produced during embryogenesis. It is often expressed in CRC, and it is the only serum marker for CRC recommended for clinical use. (Compton et al. 2000, Duffy et al. 2007). CEA was first described in 1965 (Gold et al. 1965), and modern tests are based on monoclonal antibodies. Among CRC patients, preoperative CEA is elevated in 20% (Carpelan-Holmström et al. 1995), but is not specific for CRC (Duffy et al. 2007). CEA is useful in follow-up of CRC patients, with increasing values usually a sign of recurrence/metastasis (Duffy et al. 2001, Carpelan-Holmström et al. 2004). Patients with intensive follow-up, especially with tests including CEA, have a better prognosis (Bruinvels et al. 1994, Figueredo et al. 2003). The prognostic value of CEA is not high, but it serves routinely in combination with other prognostic factors (Duffy et al. 2007).

Other serum markers such as CA19-9 have been evaluated as prognostic markers in CRC, but their significance is not clear, and they are not recommended for routine clinical use (Duffy et al. 2007).

5.8.2. Tissue biomarkers studied in this thesis

5.8.2.1. Matrix metalloproteinases

Matrix metallopreoteinases (MMPs) are zinc-dependent endopeptidases capable of degrading extracellular matrix (ECM), basement membrane (BM) proteins, and extracellular adhesions. In human beings, 24 MMPs and 4 specific MMP-inhibitors, called TIMPs, are known (Chernov et al. 2011). MMPs are secreted as proenzymes, and activated by other MMPs or serine proteinases such as plasmin, elastase, urokinase-type plasminogen-activator, or trypsin (Coussens et al. 1996). MMPs play many roles in normal biological phenomena, such as embryonal development, tissue remodelling, and angiogenesis, but also in pathological processes such as inflammation, arthrosis, and cancer. By degrading ECM and BMs, they enable tumour invasion and metastasis. In cancer progression, neovascularisation of the tumour is dependent upon MMPs (Coussens et al. 1996). MMP activity has both proapoptotic and antiapoptotic effects. Proapoptotic effects are mediated by proteolytic degradation of ECM proteins such as laminin, which acts as a ligand for cell-surface adhesion receptors, integrins. MMPs are able to cleave off the ligand of cell-death-inducing receptor Fas, a phenomenon that can result in increased or reduced apoptosis depending on physiological state (McCawley et al. 2001, Egeblad et al. 2002, Stamenkovic et al. 2003). Several MMPs associate with neoplastic diseases: MMP-1, -2, -7, -8, -9, -11, -13, and -19 (Shardella et al. 2011).

MMP-2 (gelatinase A)
MMP-2 (gelatinase A) is expressed in cancer cells, but also in surrounding stromal and immune cells (Coussens et al. 2000, Egeblad et al. 2002). It is able to degrade collagens IV and V, gelatins, and elastin. Collagen IV is the main component of basement membranes; MMP-2 can also degrade collagens I, VII, X, fibronectin, and procollagenase-3 (Coussens et al. 1996). Deficiency of MMP-2 associates in one animal model with decreased angiogenesis and tumour progression (Itoh et al. 1998). MMP-2 is able to activate growth factors and cytokines and also to inactivate adhesion molecules (McQuibban et al. 2000).

MMP-2 expression associates with aggressiveness in breast cancer (Daidone et al. 1991, Talvensaari-Mattila et al. 1998), as well as with poor prognosis (Talvensaari-Mattila et al. 2003). Expression of MMP-2 is elevated in head and neck cancer (Franchi et al. 2002). In ovarian and endometrial cancer, MMP-2 expression correlates with aggressive disease and poor prognosis (Garzetti et al. 1995, Westerlund et al. 1999, Talvensaari-Mattila et al. 2005). In prostate and bladder cancer, MMP-2 expression associates with progression and with poor disease-specific survival (Ross et al. 2003, Vasala et al. 2003). In gastric cancer, MMP-2 is a marker for poor prognosis (Allgayer et al. 1998, Mrena et al. 2006). In colorectal cancer, high MMP-2 expression has been shown to correlate with advanced stage (Levy et al. 1991) and poor prognosis (Hilska et al. 2007, Langer et al. 2008). In plasma, elevated levels of MMP-2 have been found to associate with prevalence of lymph node metastasis (Langenskiöld et al. 2005).

MMP-9 (gelatinase B)
MMP-9 (gelatinase B) is able to degrade collagens IV and V, gelatines and elastin (Coussens et al. 1996). It is overexpressed in several cancer types, such as breast (Brown et al. 1993), pulmonary (Brown et al. 1993), ovarian (Naylor et al. 1994), pancreatic (Satoh et al. 1994), prostatic (Wood et al. 1997), bladder (Monier et al. 2002), and in head and neck carcinomas (Franchi et al. 2002). It is expressed in cancer cells but also in inflammatory cells, fibroblasts, and vascular endothelium (Coussen et al. 1996). It is associated with poor prognosis in head and neck squamous cell carcinoma (Ruokolainen et al. 2004), and in non-small-cell lung cancer (NSCLC) (Peng et al. 2012). In endometrial cancer, MMP-9 correla-

tes with advanced disease and grade (Aglund et al. 2005). In gastric cancer, high MMP-9 expression associates with poor prognosis (Sier et al. 1996, Zhao et al. 2009), but a lack of association has been reported, as well (Zhang et al. 2003, Mrena et al. 2006). In early breast cancer, MMP-9 associates with improved prognosis (Scorilas et al. 2001). MMP-9 is expressed in normal colorectal tissue (Chu et al. 2012) and in colorectal adenomas; and expression correlates with grade of dysplasia (Daniel et al. 2007). The prognostic role of MMP-9 in colorectal cancer varies between different studies: an association has been reported between high MMP-9 and poor prognosis (Buhmeida et al. 2009, Chu et al. 2012), but also no association (Collins et al. 2001), as well as an association between low levels of MMP-9 and poor prognosis (Moran et al. 2005).

MMP-7

MMP-7, also called matrilysin, degrades collagens, proteoglycans, elastine, laminin, fibronectin, entactin, tenascin, and casein. It also activates other MMPs, and hence may play several roles during tissue remodelling (Chakraborti et al. 2003). MMP-7 is expressed in normal tissues, such as in monocytes, mesangial cells, endometrium, and bronchi, in ductal and glandular epithelium of the skin, in the genitourinary tract, and in gastrointestinal organs (Adachi et al. 1999, Ii et al. 2006). In cancer, MMP-7 not only plays a part in proteolysis of extracellular matrix proteins, but it also enhances tumour progression by inhibiting apoptosis of cancer cells (Wang et al. 2006), by reducing cell adhesion (Von Bredow et al. 1997), and by inducing angiogenesis (Ii et al. 2006).

MMP-7 expression is increased in many carcinomas like colorectal (McDonnell et al. 1991), gastric (McDonnell et al. 1991, Adachi et al. 1998), oesophageal (Adachi et al. 1998), pancreatic (Crawford et al. 2002, Yamamoto et al. 2001), prostatic (Pajouh et al. 1991), head and neck (Muller et al. 1991), pulmonary (Muller et al. 1991), breast (Basset et al. 1990), and hepatocellular carcinomas (Yamamoto et al. 1997). MMP-7 is also overexpressed in cancer precursor lesions such as colonic adenomas, and in premalignant lesions of the pancreas, stomach, breast, and prostate (Crawford et al. 2002). MMP-7 is expressed mainly in tumour cells- in contrast to other MMPs, which normally show stromal expression (Adachi et al. 1998, Yamamoto et al. 2001). MMP-7 expression associates with poor prognosis in gastric (Koskensalo et al. 2010), pancreatic (Yamamoto et al. 2001), and oesophageal cancers (Yamashita et al. 2000). In colorectal cancer, MMP-7 associates with advanced disease (Ishikawa et al. 1996, Adachi et al. 1999, Masaki et al. 2001, Zucker et al. 2004).

MMP-8

MMP-8, called collagenase-2, degrades collagens I, II, and III (Hasty et al. 1987) and is expressed normally in neutrophils (Weiss 1989), in bronchial and in gingival cells, and in chondrocytes (Tetlow et al. 2001, Kiili et al. 2002). MMP-8 is overexpressed in healing and non-healing cutaneous wounds (Nwomeh et al. 1999) and plays a role in degrading connective tissue in arthritic disease (Parsons et al. 1997) and in inflamed lungs in bronchiectasis (Prikk et al. 2001). MMP-8 may act as a protective agent against cancer spread by regulating tumour metastasis (Montel et al. 2004) and has been shown to be a marker for improved prognosis in breast (Gutierrez-Fernandez et al. 2008) and tongue cancers (Korpi et al. 2008). In ovarian cancer and in head and neck squamous cell carcinoma, however, MMP-8 expression associates with progression and poor prognosis (Moilanen et al. 2002, Stadlmann et al. 2003). In colorectal cancer, MMP-8 in serum associates with poor prognosis (Väyrynen et al. 2011).

5.8.2.2. Trypsinogens and TATI

Trypsinogens are zymogens for trypsin. In the gastrointestinal tract, trypsinogen is mainly produced by the pancreas; it degrades dietary proteins and activates other digestive enzymes (Paju et al. 2006).

Trypsinogen is also expressed in urogenital, vascular endothelial, and neuronal cells (Koshikawa et al. 1997, 1998). In disease states such as cancer, it is able to degrade a wide spectrum of extracellular matrix proteins (Koivunen et al. 1991) and also to activate other proteinases like MMP-1, 2, 7, 8, 9, and 13 (Sorsa et al. 1997, Lukkonen et al. 2000, Paju et al. 2001, Moilanen et al. 2003, Yamamoto et al. 2003).

Tumour-associated trypsinogen-1 and -2 are expressed in many cancer types such as colorectal (Oyama et al. 2000, Williams et al. 2001, Yamamoto et al. 2003), gastric (Fujimura et al. 1998), ovarian (Koivunen et al. 1989, Hirahara et al. 1995), pancreatic (Ohta et al. 1994), bile duct, and hepatocellular (Terada et al. 1995) and pulmonary cancers (Kawano et al. 1997). Trypsin expression associates with poor prognosis in colorectal cancer (Yamamoto et al. 2003) and trypsinogen-1 with advanced disease (Williams et al. 2001). Elevated serum trypsinogen occurs in several cancer types, such as gastric, biliary, and pancreatic (Hedström et al. 1996, 1999, 2001, Ichikawa et al. 2000).

The tumour-associated trypsin inhibitor TATI was first found in the urine of a patient with ovarian cancer (Stenman et al. 1982), but was later shown to be identical to pancreatic secretory trypsin inhibitor (PSTI) (Huhtala et al. 1982). In normal tissues, TATI is expressed in gastrointestinal and urogenital organs (Marchbank et al. 1996). In the gall bladder and stomach mucosa, TATI prevents digestion of gastric mucus, and it is expressed also in mucosa of the small intestine (Bohe et al. 1986) and colon (Fukayama et al. 1986). TATI is a specific inhibitor of trypsin (Huhtala et al. 1982), and many cancer types express both trypsin and TATI (Stenman et al. 1991). Increased TATI serum concentration correlates with poor prognosis in ovarian (Venesmaa et al. 1994, 1998, Paju et al. 2004) and bladder cancer (Kelloniemi et al. 2003). In gastric cancer, increased TATI in serum correlates with advanced stage of disease (Loizate et al. 1991, Piantino et al. 1991), whereas high tissue expression of TATI correlates with favourable prognosis (Wiksten et al. 2005). Moreover, in bladder cancer, high TATI tissue expression correlates with favourable prognosis (Hotakainen et al. 2006).

In colorectal cancer, TATI tissue expression associates with liver metastasis (Gaber et al. 2009), and strong expression correlates with advanced stage (Higashiyama et al. 1990).

5.8.2.3. EGFR

Epidermal growth factor receptor (EGFR) is a transmembrane glycoprotein of the ErbB tyrosin kinase receptor family. Ligand-receptor interaction and dimerization of the receptor leads to tyrosine autophosphorylation; this activates intracellular signal pathways such as PI3K-, Ras-MAPK-, Janus kinase- and signal transducer and activator of transcription (STAT)-pathway promoting cancer cell division and migration, inhibition of apoptosis, and angiogenesis (Cohen et al. 2003, Mendelsohn et al. 2003). In addition to EGF, many other molecules like amphiregulin, transforming growth factor (TGF) α, epiregulin, betacellulin, heparin-binding EGF, and epigen are ligands for EGFR (Saif et al. 2010). Recently, a study of panceatic cancer revealed that TATI, also called SPINK 1 or PSTI, is a ligand for EGFR. However, the affinity of TATI for EGFR is about half its affinity for EGF (Ozaki et al. 2009).

Several cancer types, like pancreatic adenocarcinomas, frequently overexpress EGFR (Oikawa et al. 1995), an overexpression that is associated with poor prognosis in lung (Ohsaki et al. 2000), breast (Sainsbury et al. 1987), ovarian (Scambia et al. 1995), bladder (Neal et al. 1990), oesophageal (Inada et al. 1999), cervical (Lee et al. 2004), and head and neck carcinomas (Hitt et al. 2005). In colorectal cancer, discordant data exist between EGFR overexpression and prognosis. In some studies, EGFR

expression associates with poor prognosis (Khorana et al. 2003, Resnick et al. 2004, Galizia et al. 2006), whereas others show no association (McKAy et al. 2002, Spano et al. 2005, Cunningham et al. 2006).

EGFR is an important target for treatment of Dukes' D or recurrent CRC. The monoclonal antibodies cetuximab and panitumumab bind to EGFR and disable the tyrosine-kinase activation and downstream signalling pathways. Nowadays, these antibodies are frequently administered as targeted therapy for colorectal cancer (Folprecht et al. 2010, Van Cutsem et al. 2011). Mutations in genes encoding molecules within an EGFR pathway can contribute to carcinogenesis and also lead to resistance to targeted therapy. KRAS mutation status is the only test in clinical use, and EGFR-targeted treatments are used only for KRAS wt tumours. Normally, activation of EGFR leads to activation of the intracellular domain, leading to KRAS signalling-cascade activation. Mutations deactivate guanosine triphosphatase (GTPase) activity, leading to accumulation of activated KRAS and also to resistance to targeted anti-EGFR therapy (Bokemeyer et al. 2009, Chang et al. 2009).

5.8.2.4. Ki-67

Ki-67 is an antigen which associates with cell proliferation. It is expressed in all other phases of the cell cycle except G0. Ki-67 expression reflects cell proliferation and the growth potential of the tumour (Endl et al. 2000). In CRC, Ki-67 associates with tumour differentiation, metastasis, and local invasiveness (Ishida et al. 2003, Valera et al. 2005). Ki-67 can also associate with better outcome; overexpression associates with complete response to chemoradiotherapy in oesophageal cancer (Ressiot et al. 2008). For colorectal cancer, results reported have been divergent. Ki-67 has associated with poor prognosis (Palmqvist et al. 1999), but also with improved survival (Allegra et al. 2003), or it has had no prognostic value at all (Buglioni et al. 1999).

5.8.2.5. p53

TP53 is a tumour-suppressor gene, and its mutations can occur in about 50% of CRC cases. These mutations play an important role in carcinogenesis (Steele et al. 2005). The translational product of the TP53 gene is a nuclear phosphoprotein acting as a transcription factor, and mutation in its gene leads to accumulation of mutated protein p53 in nuclei, with resultant disturbances in apoptosis and angiogenesis, and in cell-cycle and genomic maintenance (Baas et al. 1994, Vogelstein et al. 2000, Mills et al. 2005). The mutated TP53 gene can be detected by sequence analysis (Munro et al. 2005). The mutated protein is more stable than is wild type p53, and is detectable by immunohistochemistry (Levine et al. 1997).The p53 mutation appears in several malignancies (Hollstein et al. 1991), and overexpression of p53 in tissues associates with poor prognosis in gastric cancer (Victorzon et al. 1996, Mrena et al. 2010). Mutation in the p53 gene leads to increased risk of death and failure to respond to radiation in rectal cancer (Munro et al. 2005), and overexpression of mutated protein associates with poor prognosis in CRC (Manne et al. 1997, Kaklamanis et al. 1998). Loss of p53 function is a late event in the adenoma-carcinoma sequence (Fearon and Vogelstein 1990), and a predominance of mutations appears in rectal and distal colonic tumours (Hamelin et al. 1994, Goh et al. 1999). p53 is not in clinical use as a predictive marker in CRC (Duffy et al. 2007), but patients with wild-type TP-53, unlike those with mutant TP53, gain a survival benefit from FU treatment (Iacopetta 2003).

6. AIMS OF THE STUDY

The purpose of this study was to evaluate the prognostic roles of tissue expression of matrix metalloproteinases 2, 7, 8, and 9 , and of trypsinogens, TATI , EGFR, p53, and Ki-67 in colorectal cancer. Particularly the aim was to discover markers to define patients with Dukes' B carcinoma and poor prognosis who would benefit from adjuvant therapy.

The specific aims were to assess the prognostic role of:

- MMP-7, MMP-2, MMP-8 and MMP-9
- TATI and its coexpression with trypsinogen-1 and trypsinogen-2
- EGFR and EGFR coexpression with TATI

7. PATIENTS AND METHODS

7.1. Patients (I-V)

The study included 643 consecutive patients who underwent surgery for colorectal cancer at the Department of Surgery, Meilahti Hospital, Helsinki University Central Hospital, between 1982 and 1998. Of these, 9 patients were excluded for wrong final diagnosis and 9 for synchronous multiple tumours; 2 cases were excluded because they were not operated and therefore only biopsy specimen was available. In addition, varying number of cases were excluded due to an insufficient archival tissue specimen in each marker study. Finally, 623 cases remained, 333 of them male.

Tumours were staged according to the modified Dukes' classification (Davis et al 1984). Of the tumours, 92 were Dukes' A, 224 Dukes' B, 162 Dukes' C, and 145 Dukes' D. Median age was 67.3 years (range 22.7-90.3), and median follow-up time 4.85 years (range 0-24.7). Median follow-up time for survived patients (n=137) until March 2011 was 16.2 years (range 12.9-25.8).

For MMP-9, a validation series included 213 patients, 131 of them male, treated between 1998 and 2001. Of these tumours, 31 were Dukes' A, 70 Dukes' B, 69 Dukes' C, and 41 Dukes' D. Mean age was 66.2 years (range 27.6-98.6), and median follow-up time 5.93 years (range 0.003-13.2). Of the tumours, 7 were WHO grade I, 161 grade II, 37 grade III, and 4 grade IV.

Clinical data (age, Dukes' stage, gender, WHO grade, tumour histology, and location) were retrieved from patient records, and survival data and cause of death from the Population Register Centre of Finland and Statistics Finland. The follow-up was until March 2009 for Studies I and III, March 2011 for Studies II and IV, and October 2012 for Study V.

The study was approved by the local Ethics Committee (Dnro HUS 226/E6/06) and the National Supervisory Authority for Welfare and Health (Dnro 3990/04/046/07).

7.2. Tissue specimens (I-V)

Formalin-fixed and paraffin-embedded surgical tissue samples were collected from the archives of the Department of Pathology, University of Helsinki. All routine slides were re-evaluated. Histopathologically representative regions of tumour specimens were defined and marked on H&E slides. Three 1.0-mm cores from each tumour block were sampled with a semiautomatic tissue microarrayer (Tissue Arrayer 1, Beecher Instruments Inc., Silver Spring, MD, USA). Three series of tissue microarray (TMA) blocks were constructed, each including one sample from each patient. From each block, 4-µm slides were cut and processed for immunohistochemistry.

7.3. Immunohistochemistry (I-V)

The Lab Vision Autostainer TM 480 (LabVision, Fremont, CA, USA) served for immunohistochemistry, except for MMP-7, TATI, and trypsinogen-2 stainings. Specimens were deparaffinized in xylene and rehydrated through graded alcohol series. The pretretment and immunostaining are presented in Table 3. Meyer's haematoxylin served for counterstaining, followed by a 10-minute wash in tap water and mounting in aqueous mounting medium (Aquamount, BDH, Poole, UK) (Table 3).

Table 3. Characteristics of immunohistochemical staining and cut-off values for tumour markers.

Tumour marker	Antibody	Dilution	Manufacturer	Amplification kit	Pre-treatment	Cut-off
MMP-2	MS-806-P0	1:700	NeoMarkers	ChemMate EnVision det system	PTM 20 min in 98ºC	≥ score 1 cytoplasmic staining
MMP-7	MAB3315	1:2000	Chemocin Laboratories	Vectastain ABC	Microwawe 700W	≥ 50% stained cytoplasma
MMP-8	ref (Sorsa)	1:100	ref(Sorsa)	ChemMate EnVision system	t-hcl/PTM 20 min in 98ºC	≥ score 1 cytoplasmic staining
MMP-9	RB-1539-R7	1:1500	NeoMarkers	ChemMate EnVision det system	PTM 20 min in 98ºC	≥ score 1 cytoplasmic staining
TATI	6E8	1:500	ref (Osman)	ChemMate EnVision det system	trypsin 30 min in 37 ºC	≥ score 1 cytoplasmic staining
Trypsinogen-1	MAB1482	1:500	Chemicon	ChemMate EnVision det system	t-hcl/PTM 20min in 98ºC	≥ score 1 cytoplasmic staining
Trypsinogen-2	8F7	1:200	ref (Itkonen)	ChemMate EnVision det system	pepsin 20 min in 37 ºC	≥ score 1 cytoplasmic staining
EGFR	NCL-EGFR clone EGFR.113	1:10	Novocastra	AdVance	TE/PTM 60 min, 98ºC	≥ score 1 cytoplasmic staining
Ki-67	clone MIB-1	1:100	Dako	ChemMate EnVision det system	TE/PTM 20 min in 98ºC	> 10% stained nuclei
p53	DO7	1:50	Dako	ChemMate EnVision det system	TE/PTM 20 min in 98 ºC	> 10% stained nuclei

7.4. Scoring (I-V)

Immunohistochemical stainings were evaluated by two independent investigators (S.K. and S.N. or S.K. and J.H.) without knowledge of clinical data. When they differed regarding values, the consensus score was determined. Spots without cancer cells were excluded. The highest of three values was used for each patient.

Cytoplasmic MMP-7 immunoreactivity was evaluated by percentage of stained cells. More than 50% was scored as 3, 10 to 50% as 2, and less than 10% as 1. Negative-stained samples were scored as 0. For further analysis, patients were divided into two groups, low positivity (score 0-2) or strong positivity (score 3).

Cytoplasmic MMP-2, MMP-8, and MMP-9 immunoreactivity was evaluated by intensity of stained cells: strongly stained cells were scored as 3, moderately stained as 2, and weakly stained as 1. Absence of positivity was scored as 0. For final analysis, the patients were divided into two groups, negative (score 0) and positive (scores 1-3) immunoreactivity.

Cytoplasmic TATI, trypsinogen-1, and trypsinogen-2 immunopositivity was scored like MMP-2, MMP-8, and MMP-9, and for final analysis, the patients were divided into two groups, negative (score 0) and positive (score 1-3) immunoreactivity.

Cytoplasmic EGFR immunopositivity was scored like MMP-7. For further analysis, patients were divided into two groups, with negative (score 0) and positive (scores 1-3) immunoreactivity.

Nuclear Ki67 and p53 positivity were evaluated: 1 to 10% was scored as 1, 11 to 50% as 2, and more than 50% as 3. Negative staining was scored as 0. For further analysis, patients were divided into two groups, negative (score 0-1) and positive (score 2-3) immunoreactivity.

7.5. Statistical analysis (I-V)

The correlation between staining score and clinicopathological variables was assessed with the $\chi 2$ test or Fisher´s exact test when applicable. The Mann-Whitney U-test allowed determination of the correlation between age and staining score. Life-tables were calculated by the Kaplan-Meier method. Significance of the difference between groups was assessed with the log-rank test or log-rank test for trend. Patients alive at the end of follow-up and patients who died from unrelated causes or within 30 days after the operation were treated as censored cases. The Cox proportional hazards model served for multivariate survival analysis. Clinical variables included in the model as covariates were age, Dukes' stage, differentiation (WHO grade), tumour location (colon or rectum), and tumour histology (adenocarcinoma or mucinous carcinoma). The likelihood ratio test was applied for exclusion or inclusion of significant variables. A p-value of 0.05 was considered significant. Statistical analyses were performed with SPSS 17.0 software.

8. RESULTS

8.1. Immunohistochemical expression of tissue markers

Immunohistochemical expression of samples is presented in Table 4. Cytoplasmic immunoexpression was observed for MMP-2, MMP-7, MMP-8, MMP-9, TATI, trypsinogen-1, trypsinogen-2, and EGFR, whereas cell nuclei were negative. Nuclear immunoexpression was observed for Ki-67 and p53, whereas cytoplasma was negative.

Table 4. Immunohistochemical expression of tumour markers in colorectal cancer.

Marker	Patients (n)	Score 3 (%)	Score 2 (%)	Score 1 (%)	Score 0 (%)
MMP-2	581	56 (9.6)	98 (16.9)	171 (29.4)	256 (44.1)
MMP-7	545	105 (19.3)	103 (18.9)	134 (24.9)	203 (37.2)
MMP-8	548	39 (7.1)	153 (27.9)	237 (43.2)	119 (21.7)
MMP-9	581	50 (8.6)	108 (18.6)	192 (33.0)	208 (35.8)
TATI	569	72 (12.7)	126 (22.0)	173 (30.4)	198 (34.8)
Trypsinogen-1	581	61 (10.9)	102 (18.0)	213 (38.0)	185 (33.0)
Trypsinogen-2	549	73 (13.3)	120 (21.9)	266 (48.5)	90 (16.4)
EGFR	520	69 (13.3)	172 (33.1)	236 (45.4)	43 (8.3)

Marker	Patients (n)	Score 2-3 (%)	Score 0-1 (%)
Ki-67	479	69 (14.4)	410 (85.6)
p53	503	74 (14.7)	429 (85.3)

In the validation series, 167 (78.4%) of 213 cases were MMP-9 positive. In 12 (5.6%) patients, the immunopositivity was scored as 3, in 41 (19.2%), as 2, and in 114 (68.3%), as 1.

For analysis of EGFR and TATI coexpression, 511 samples were available. EGFR+ TATI+ immunoexpression was apparent in 321 (62.8%), EGFR+ TATI- in 151 (29.5%), EGFR-TATI + in 25 (4.9%), and EGFR-TATI- in 14 (2.7%).

For rectal cancer, 74 patients had been treated with preoperative short-course radiotherapy, which, however, did not affect the EGFR expression correlated with tumours without radiotherapy (data not shown).

8.2. Associations of different variables

8.2.1. Association of tumour markers and clinicopathological variables (I-V)

Association of tumour markers and clinicopathological variables is shown in Table 5. Clinicopathological variables were age, gender, Dukes' stage, WHO grade, histology, and location (colon/rectum).

MMP-9 correlated with differentiation ($p < 0.001$); it was more often positive in high- to moderately differentiated tumours than in poorly differentiated tumours. In the validation series, no correlation between clinicopathological variables was detected.

Table 5. Association of tumour markers with clinicopathological variables.

Tumour marker	Age	Gender	Dukes´stage	WHO grade	Histology	Location
MMP-2	NS	NS	NS	NS	NS	NS
MMP-7	NS	NS	NS	$p < 0.001$	NS	NS
MMP-8	NS	NS	NS	NS	NS	NS
MMP-9	NS	NS	NS	$p < 0.001$	NS	NS
TATI	NS	NS	NS	NS	$p < 0.001$	NS
Trypsininogen-1	NS	NS	$p = 0.045$	NS	NS	NS
Trypsinogen-2	NS	NS	$p = 0.050$	$p = 0.012$	NS	NS
EGFR	NS	NS	NS	$p = 0.040$	NS	NS
Ki-67	NS	NS	NS	$p = 0.032$	NS	NS
p53	NS	NS	NS	NS	NS	$p = 0.021$

Trypsinogen-1 positivity correlated with Dukes' stage ($p = 0.045$); the proportion of positive tumours was lower in metastasized (Dukes' C-D) than in local (Dukes' A-B) disease. Trypsinogen-2 positivity correlated with high Dukes' stage ($p = 0.050$) and differentiation ($p = 0.012$).

TATI positivity associated significantly with histological type of adenocarcinoma ($p < 0.001$) and inversely with differentiation ($p < 0.001$).

EGFR immunoreactivity correlated with tumour grade and was more often positive in high and moderately differentiated tumours ($p = 0.040$).

EGFR and TATI coexpression correlated with histology, and was prominent in adenocarcinomas ($p = 0.005$). Moreover, EGFR+TATI+ correlated with differentiation and was more frequent in highly and moderately differentiated tumours ($p < 0.001$).
p53 immunoreactivity associated with location, and was more often positive in rectal than in colon tumours ($p = 0.021$). Ki-67 associated inversely with grade ($p = 0.016$).

8.2.2. Associations between markers

When associations between expression of markers were analyzed, a significant association appeared between MMP-7 and MMP-8 ($p < 0.001$), MMP-7 and MMP-9 ($p = 0.008$), and between MMP-8 and MMP-9 ($p < 0.001$) expressions.
A significant association appeared between TATI and MMP-8 ($p = 0.027$) and TATI and MMP-9 ($p = 0.009$), and also between trypsinogen-2 and MMP-8 ($p < .001$), and between trypsinogen-2 and MMP-9 ($p = 0.006$).
A significant association appeared also between EGFR and trypsinogen-2 ($p = 0.005$), EGFR and MMP-8 ($p = 0.022$), and EGFR and MMP-9 ($p < 0.001$).

8.2.3. Survival analysis (I-V)

The cumulative, disease-specific 5-year survival was 54.9% in rectal cancer, and 57.9% in colon cancer. The relationship between preoperative characteristics and survival is presented in Table 6, and between tumour marker expression and survival analysis of markers in Table 7.

Table 6. Univariate analysis of the relationship between clinicopathological variables and survival in 623 colorectal cancer patients.

Clinicopathological variable	Patients	Cumulative 5-year survival %	χ2	p-value
Age			8.548	0.003
<65 years	259	61.6		
≥65 years	364	52.7		
Gender			0.006	0.940
Female	290	55.5		
Male	333	57.2		
Dukes' stage			293.88	<0.001
A	92	88.0		
B	224	77.4		
C	162	50.1		
D	145	7.9		
Differentiation (WHO grade)			18.554	<0.001
1	19	76.7		
2	408	60.6		
3	166	45.9		
4	28	36.7		
missing	2			
Histologic type			1.711	0.191
Adenocarcinoma	544	57.9		
Mucinous carcinoma	79	46.4		
Tumor location			2.061	0.151
Colon	341	57.9		
Rectum	279	54.9		
missing	3			

Table 7. Univariate analysis of the relationship between tumour marker expression and survival in colorectal cancer patients.

Tumour marker	5-year survival %	χ2	p-value
MMP-2		0.507	0.477
negative	58.8		
positive	56.0		
MMP-7		0.973	0.324
low	59.5		
high	52.5		
MMP-8		0.507	0.477
negative	56.6		
positive	58.8		
MMP-9		5.923	0.015
negative	52.2		
positive	62.5		
TATI			
negative	48.3	6.725	0.010
positive	63.0		
Trypsininogen-1		0.900	0.343
negative	53.9		
positive	58.4		
Trypsinogen-2		0.08	0.784
negative	56.0		
positive	57.7		
EGFR		7.549	0.006
negative	40.5		
positive	59.9		
Ki-67		2.496	0.114
low	55.4		
high	67.4		
p53		0.984	0.321
low	59.1		
high	53.1		

8.2.3.1. Metalloproteinases (I, IV)

In univariate analysis, MMP-2 expression did not associate with survival ($p = 0.477$, $\chi2 = 0.507$). High MMP-7 immunoexpression associated with poor 5-year survival ($p = 0.028$), but during long-term (24.7 years) follow-up, the difference in survival between patient groups disappeared ($p = 0.308$, $\chi2 = 0.973$; Figure 2). The difference in 5-year survival appeared for colonic, but not for rectal tumours. MMP-8 did not associate with survival ($p = 0.719$, $\chi2 = 0.129$). MMP-9 immunoexpression associated inversely with survival ($p = 0.015$, $\chi2 = 5.923$; Figure 3). In subgroup analysis, an association between improved survival and MMP-9 immunoexpression existed only for Dukes' B tumours, but it was such a strong prognostic factor that it affected the results of the whole group ($p = 0.018$). In the validation series of MMP-9, 5-year survival was 64.8% in MMP-9-positive patients, 63.5% in MMP-9-negative patients; the difference was not significant ($p = 0.418$).

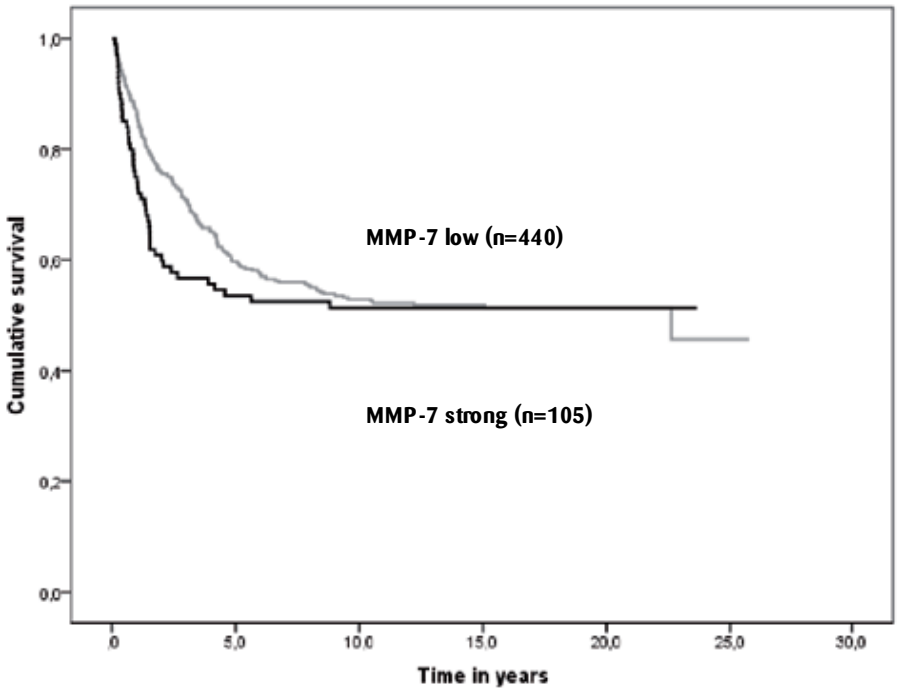

Figure 2. Survival Curves of MMP-7 immunoexpression in colorectal cancer patients.

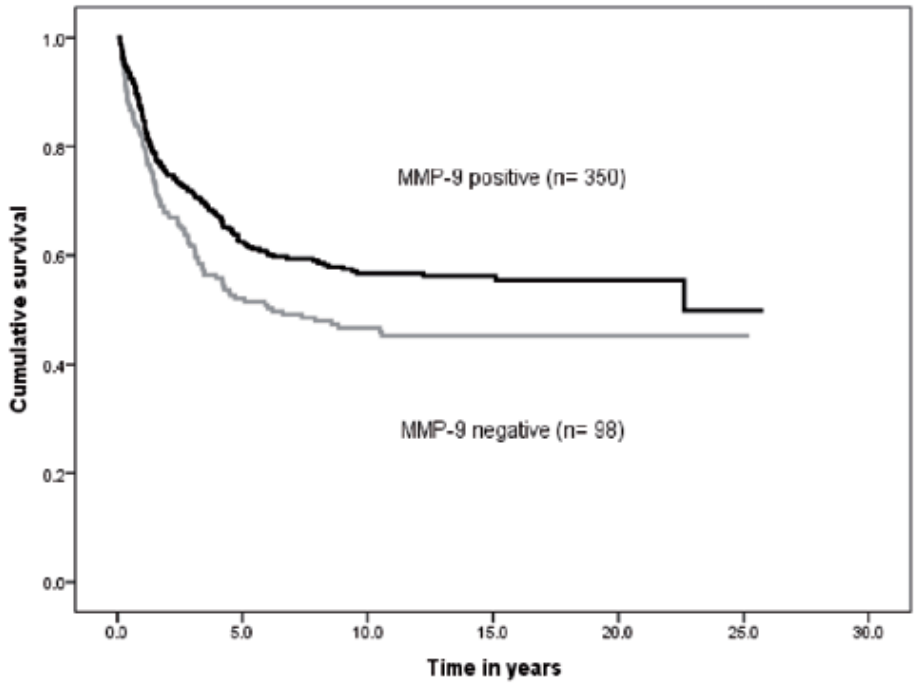

Figure 3. Survival curves of MMP-9 immunoexpression in colrectal cancer patients.

8.2.3.2. Trypsinogen-1, trypsinogen-2, and TATI (III)

TATI immunoreactivity associated inversely with survival ($p = 0.010$, $\chi 2 = 6.503$). Disease-specific 5-year survival for patients with TATI-positive tumours was 63.0% compared to 48.3% for those negative for TATI (Figure 4). Moreover, in tumours with lymph node (Dukes' C) or distant metastasis (Dukes' D), high TATI positivity associated with better survival: 38.0% in patients with positive- compared to 19.4% in those with TATI-negative tumours ($p = 0.013$). For trypsinogen-1, 5-year survival was 58.4%

in immunopositive, and 53.9% in negative groups (p = 0.343, $\chi 2 = 0.902$), and for trypsinogen-2, 5-year survival was 57.7% and 56.0% (p = 0.784, $\chi 2 = 0.255$).

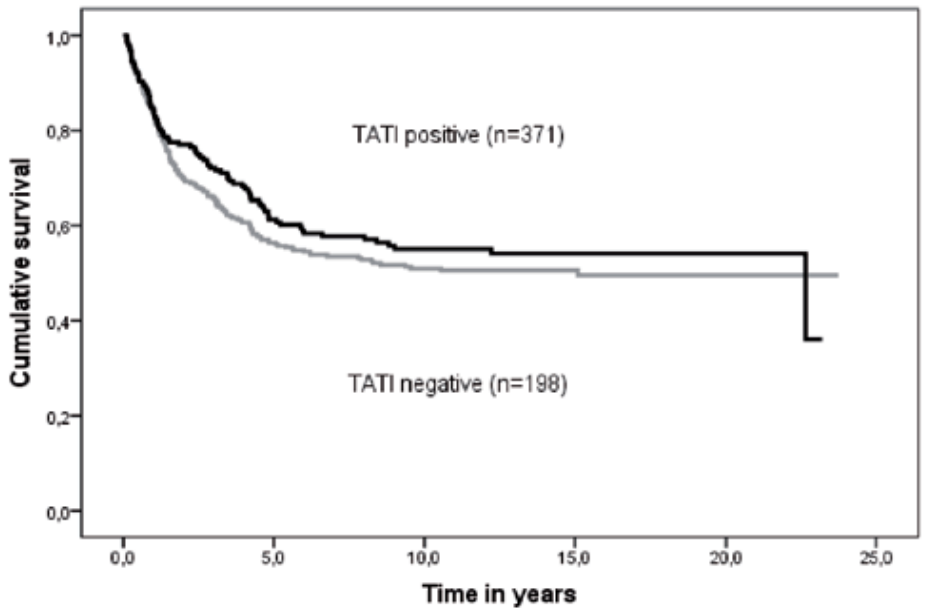

Figure 4. Survival curves of TATI immunoexpression in colorectal cancer patients.

In subgroup analyses of trypsinogen-1- and trypsinogen-2-positive tumours, low TATI-positivity associated significantly with improved survival (p = 0.004, $\chi 2 = 8.19$; p = 0.002, $\chi 2 = 9.407$).

8.2.3.3. EGFR (V)

In univariate analysis, EGFR immunoexpression (p = 0.006, $\chi 2 = 5.436$), age (p = 0.009), WHO grade (p < 0.001), and Dukes' stage (p < 0.001) associated with prognosis. Five-year survival was 59.9% in EGFR-positive patients compared to 40.5% in EGFR-negative patients. In subgroup analysis of colon versus rectal tumours, significantly better survival appeared in EGFR-positive patients than in EGFR-negative ones (p = 0.001) only in rectal cancers, (Study V, data not shown).

Concomitant expression of EGFR and TATI associated with prognosis. Five-year survival was 65.4% in EGFR+ TATI+ patients, 48.5% in EGFR+ TATI-, 43.2% in EGFR- TATI +, and 42.4% in EGFR- TATI- patients (p < 0.001) (Figure 5).

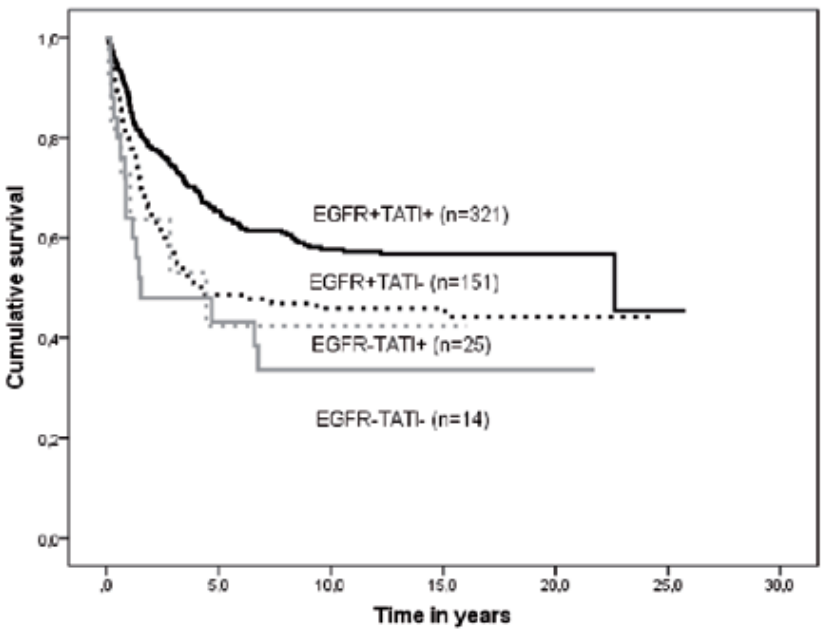

Figure 5. Survival curves of concomitant immunoexpression of EGFER and TATI colorectal cancer patients.

8.2.3.4. p53 (II)

p53 did not associate with survival ($p = 0.32$, $\chi 2 = 0.984$). Five-year survival was 59.1% in patients with low p53 expression, and 53.1% in patients with strong p53 immunoreactivity.

8.2.3.5. Ki-67 (II)

Ki-67 did not associate with survival ($p = 0.114$, $\chi 2 = 2.496$). Five-year survival was 55.4% in patients with low Ki-67 expression and 67.4% in patients with strong Ki-67 expression.

8.2.4. Multivariate analysis (I-V)

In Study I, MMP-7 was not an independent prognostic factor, but Dukes' stage ($p < 0.001$) and tumour location ($p = 0.014$) were independent prognostic factors; advanced stage and location in the rectum associated with poor prognosis.

In Study II, neither Ki-67 ($p = 0.662$) nor p53 ($p = 0.769$) was an independent prognostic factor.

In Study III, low TATI immunoexpression ($p = 0.044$), in addition to age ($p < 0.001$), Dukes' stage ($p < 0.001$), differentiation ($p = 0.042$), and tumour location ($p = 0.008$), was an independent prognostic factor: low TATI, older age, advanced stage, poor differentiation, and location in the rectum associated with poor prognosis. Neither histological type nor trypsinogen-1 nor trypsinogen-2 tissue expression provided significant prognostic information. Moreover, TATI positivity was an independent prognostic factor both in trypsinogen-1-positive tumours ($p = 0.007$) together with age ($p < 0.001$), Dukes' stage ($p < 0.001$), and location ($p = 0.047$), and in trypsinogen-2-positive tumours ($p = 0.006$) together with age ($p < 0.001$) and Dukes' stage ($p < 0.001$).

In Study IV, age ($p < 0.001$), Dukes' stage ($p < 0.001$), location ($p = 0.016$), and differentiation ($p = 0.005$) were independent prognostic factors, but not MMP-9; older age, location in rectum, and poor differentiation associated with poor prognosis. In the subgroup of Dukes' B tumours, MMP-9 positivity was an independent prognostic factor ($p = 0.037$), as was tumour location ($p = 0.042$); MMP-9 associated with better prognosis.

In Study V, EGFR ($p = 0.023$), patient's age ($p < 0.001$), Dukes' stage ($p < 0.001$), tumour location ($p = 0.001$), and WHO grade ($p = 0.033$) were independent prognostic factors: low EGFR expression, older age, advanced stage, location in rectum, and high grade associated with poor prognosis. In analysis of concomitant expression, EGFR+TATI+ ($p < 0.001$), age ($p < 0.001$), Dukes' stage ($p < 0.001$), and location ($p = 0.003$) were independent prognostic factors; concomitant EGFR and TATI expression associated with improved prognosis.

Finally, multivariate analysis of all prognostic clinicopathological variables and individual tumour markers showed that age ($p < 0.001$), Dukes' stage ($p < 0.001$), location in rectum ($p = 0.006$), WHO grade ($p = 0.020$), and TATI ($p = 0.044$) were independent prognostic markers (Table 8). Furthermore, multivariate analysis was performed, including EGFR-TATI coexpression as a tumour marker. Age ($p < 0.001$), Dukes' stage ($p < 0.001$), and EGFR-TATI co-expression ($p = 0.001$) were independent prognostic markers.

Table 8. Cox multivariate survival analysis of 463 colrectal cancer patients.

Covariate		Wald statistic	p-value	RH	95% CI
Age		33.710	<0.001	1.032	1.021-1.043
Dukes' stage					
A		302.623	<0.001		
B		2.380	0.123	1.630	0.876-3.033
C		30.357	<0.001	5.347	2.945-9.707
D		123.502	<0.001	29.693	16.328-53.998
Differentiation (WHO Grade)					
	1	9.802	0.020		
	2	3.248	0.071	2.500	0.923-6.773
	3	5.043	0.025	3.227	1.116-8.973
	4	6.920	0.009	4.536	1.470-13.993
Tumour location in rectum		7.684	0.006	1.433	1.1111-1.848
Histologic type			NS		
MMP-2			NS		
MMP-7			NS		
MMP-8			NS		
MMP9			NS		
TATI		4.050	0.044	0.759	0.580-0.993
Trypsinogen-1			NS		
Trypsinogen-2			NS		
EGFR			NS		
p53			NS		
Ki67			NS		

NS= not significant, RH= relative hazard, CI= confidence interval at 95% level

9. DISCUSSION

In CRC, stage is the most important prognostic factor, and adjuvant treatment both reduces the recurrence and improves prognosis. Dukes' C/Stage III patients usually receive adjuvant treatment. About 20% of Dukes' B/Stage II patients have a recurrence and will die from CRC, and adjuvant treatment is recommended for patients with known risk such as perineural or vascular invasion, perforation, or T4 tumour. More prognostic tools are crucial to identify high-risk patients. This study identified several prognostic markers which may help in clinical decision-making. Positivity for MMP-9 emerged as a marker for improved prognosis in Dukes' B CRC, but strong positivity for MMP-7 was a marker for poor 5-year survival. TATI positivity and especially TATI and EGFR co-expression were markers for improved survival.

9.1. Tumour markers

9.1.1. Metalloproteinases

The prognostic role of MMP-2 in CRC prognosis is unclear. In this study, MMP-2 positivity correlated neither with clinicopathological variables nor with survival These results are in concordance with those of several other studies (Schwandner et al. 2007, Unsal et al. 2008, Hong et al. 2011). On the other hand, MMP-2 immunoexpression has been reported to associate with advanced CRC disease (Levy et al. 1991, Papadopoulou et al. 2001, Matsuyama et al. 2002), and high expression of MMP-2 in cancer cells and stroma associates with poor prognosis (Matsuyama et al. 2002, Hilska et al. 2007). MMP-2 expression in tumour cells and stroma are comparable in 87% of the cases (Hilska et al. 2007). In this study, stromal expression was not evaluated because it could not be reliably analyzed from TMA samples. One explanation for varying results can be differences in methods. MMP-2 is predominantly expressed in the tumours border area (Hong et al. 2011), and in this respect, analysis of TMA stainings is less reliable than is analysis of whole-tissue sections.

MMP-8 plays a protective role in cancer, one linked to its ability to regulate the carcinogen-induced inflammatory response rather than to its collagenase function (Montel et al. 2004, Gutierrez-Fernandez et al. 2008). In breast cancer, MMP-8 reduces metastatic potential in vitro (Montel et al. 2004, Decock et al. 2008). In melanoma and lung cancer, MMP-8 is able to inhibit metastasis formation by modulating cancer cell invasion and adhesion (Gutierrez-Fernandez et al. 2008). In serum, MMP-8 correlates positively with advanced stage of CRC (Väyrynen et al. 2011), but we expected that tissue expression of MMP-8 could serve as a marker for improved prognosis. MMP-8 immunoreactivity appeared in 78.3% of samples but lacked any correlation with clinicopatholical variables or with survival.

MMP-9 expression associated with good prognosis in Dukes' B colorectal cancer. At the other stages, no association with survival emerged. In Dukes' B, the difference was so great that it affected the results of the whole cohort. In the validation series, the results differed, and no association between MMP-9 expression and survival appeared. One reason for differing results between cohorts can be the changed surgical, pathological, and oncological treatments. The TME technique is used in rectal cancer, more lymph nodes are examined, leading to stage migration, and patients more usually receive

chemotherapy. Results in Study IV differ from those of a recent study of stage II CRC, in which high MMP-9 expression associated with higher recurrence rate, shorter disease-free survival, and also with shorter disease-specific survival; the association with disease-specific survival was not significant in multivariate analysis, however (Buhmeida et al. 2009). In CRC patients, high MMP-9 expression has been associated with liver metastasis (Koumura et al. 1997), and elevated MMP-9 mRNA levels with poor disease-free and overall survival (Zeng et al. 1996). As in the validation series, many studies have failed to show, in CRC, any correlation between immunohistochemical expression of MMP-9 and survival or clinicopathological parameters (Masuda et al. 1999, Collins et al. 2001, Roca et al. 2006, Jensen et al. 2010). Moran reported findings similar to those of Study IV, noticing a low level of MMP-9 immunoexpression to be correlated with poor prognosis (Moran et al. 2005).

In many other cancers: lung cancer, head and neck squamous cell carcinoma, and gastric cancer, MMP-9 associates with poor prognosis (Brown et al. 1993, Sier et al. 1996, Ruokolainen et al. 2004), but in early breast cancer, surprisingly, elevated MMP-9 associates with better prognosis (Scorilas et al. 2001). In cancer, host responses such as intra- or peritumoral inflammation reaction or desmoplasia predict better prognosis (Ropponen et al. 1997, Galon et al. 2006, Crispino et al. 2008).

Some matrix-degrading proteinases may play a defensive role by supporting the local immune/inflammatory response, at least in theory. In CRC, stromal expression of MMP-9 inversely associates with liver metastasis and tumour infiltration (Takeha et al. 1997), and in Dukes' B and C colorectal cancer patients, stromal MMP-9 positivity also inhibits metachronous haematogenic metastasis (Saito et al. 2000). In the present study, stromal MMP-9 expression was not evaluated, because the TMA were constructed with punches taken from cancerous regions, making them unsuitable for reliable stroma evaluation.

The proposed role of MMP-9 immunoexpression in colorectal cancer is dual; it plays a role in matrix degradation enabling tumour invasion, but it also seems to be a supportive factor for host-defensive mechanisms against cancer spread. Clinically, MMP-9 may serve as a useful prognostic tool in identifying those MMP-9-negative Dukes' B patients with poor prognosis who may benefit from adjuvant treatment.

MMP-7 plays a role in CRC development and progression (Zucker et al. 2004) and is important in the growth of early colonic adenomas and their transformation into invasive cancer (Newell et al. 1994). In vitro, MMP-7 induces metastasis (Kioi et al. 2003). In this study, contrary to some other reports (Ishikawa et al. 1996, Adachi et al. 1999, Masaki et al. 2001, Zucker et al. 2004), no correlation emerged between high MMP-7 immunoexpression and lymph node or distant metastasis, even though a correlation appeared between MMP-7 and tumour differentiation.

MMP-7 expression was a marker for poor 5-year outcome, but in long-term follow-up, the difference between patients with high and low MMP-7 expression decreased. This phenomenon has been described for some other markers in other cancer types, such as in breast cancer and soft tissue sarcomas (Hilsenbeck et al. 1998, Engellau et al. 2001, Leivonen et al. 2001a, 2001b, Railo et al. 2007). In this study, MMP-7 was not an independent prognostic marker.

Here, MMP-7 expression was less often positive than earlier reported (Newell et al. 1994, Adachi et al. 1999, Zucker et al. 2004) which may be due to differences in staining technique and in antibodies. It may also be due to the use of TMAs. Since MMP-7 is said to be more strongly expressed in the invasive front of a tumour, some focal positivity may have been missed.

MMP-7 inhibition by an antisense expression vector or by antisense nucleotides suppress the in vitro invasiveness and in vivo metastatic potential of pancreatic cancer and Ewing's sarcoma cells (Wang et al. 2006). Clinical use of MMP-7 inhibitors, because of their poor therapeutic effect and problems with side-effects, has been disappointing, (Mimori et al. 2004). The tyrosine kinase inhibitor gefitinib and its derivatives, known to be effective against MMP-7-positive tumor cells, have undergone clinical testing in colon cancer patients (Mimori et al. 2004).

9.1.2. TATI and trypsinogens

Study II assessed the prognostic value of TATI tissue expression in CRC and found it to be an independent prognostic marker in multivariate analysis. TATI is an inhibitor of trypsin, but neither trypsinogen-1 nor trypsinogen-2 tissue expression correlated with prognosis. Furthermore, in both trypsinogen-1-positive and trypsinogen-2-positive tumours, positive TATI expression was an independent prognostic marker in multivariate analysis). In advanced disease (Dukes' C and D), TATI-positive tumours had better prognosis than did negative tumours, supporting the theory that TATI plays a role in suppressing cancer spread (Wiksten et al. 2005). When co-expressed with tumour-associated trypsin, TATI may therefore protect tissue from destruction and thereby from tumour spread (Stenman et al. 1990, 1991). In concordance with previous findings, TATI positivity correlated inversely with WHO grade; its expression was higher in well-differentiated carcinomas (Higashiyama et al 1990), and TATI was more often expressed in adenocarcinomas than in mucinous carcinomas (Higashiyama et al. 1990). Still, TATI expression showed no correlation with Dukes' stage, also in concordance with others' findings (Higashiyama et al. 1990). A significant correlation is observable in CRC between TATI expression and liver metastases (Gaber et al. 2009).

Elevated TATI concentration in serum is associated with poor prognosis in several cancer types (Loizate et al. 1991, Piantino et al. 1991, Venesmaa et al. 1994, 1998, Kelloniemi et al. 2004, Paju et al. 2004, Gaber et al. 2010). This probably reflects aggressive tumour behaviour, with basement membrane and extracellular matrix (ECM) destruction leading to leakage of tumour-associated proteins into the circulation. No discrepancy thus occurs between results for the prognostic value of high TATI in tissue and in serum.

A correlation exists between low TATI tissue expression and advanced tumour, lymph node metastasis, and stage (Gaber et al. 2009), in concordance with our results. This was not unexpected, since Gaber et al. used the same antibody as ours and similar staining techniques. More surprising is that despite their correlation between low TATI expression and predictors of poor prognosis, they found a correlation between low TATI and better overall survival (Gaber et al. 2009). The reason for this discrepancy is difficult to explain. In this study, low TATI expression correlated with factors reflecting poor prognosis such as stage and differentiation, and consequently also with worse disease-specific survival.

Trypsinogen-1 immunoexpression correlated inversely with Dukes' stage. Contrary to our current results, trypsin has been shown to correlate with advanced TNM stage and short survival (Yamamoto et al. 2003). However, trypsin positivity was analyzed from the invasive front of the tumour, with the cut-off applied being different from ours (Study II). Trypsinogen-1 failed to correlate with any other clinicopathological variable, in concordance with others' data (Oyama et al. 2000). No correlation appeared here, between tissue expression of trypsinogen-1, trypsinogen-2, and TATI, in discordance with earlier data (Solakidi et al. 2003).

It seems likely that TATI has various functions in malignancies. TATI is co-expressed with tumour-associated trypsin, and it seems apparent that TATI plays a role as an inhibitor of trypsin and possibly of other serine proteinases. Trypsin, on the other hand, has been shown to activate metalloproteinases; and these, together with serine proteinases, play a role in degradation of basement membranes and tissue matrix, thereby facilitating local tumour invasion (Coussens et al. 1996). The role of TATI as a trypsin inhibitor could explain the correlation between high TATI tissue expression and better prognosis. On the other hand, TATI promotes tumour invasion and enhances tumour cell survival and metastasis both in vitro and in vivo through trypsin-independent mechanisms. This occurs by its regulating several oncogenic pathways (Goyer et al. 2008).

It is possible that, among cancers, the role of TATI varies. The role of TATI expressed together with EGFR may be important in the development of pancreatic cancer (Ozaki et al. 2007), but in colorectal cancer, its role of inhibiting trypsin may be dominant. On the other hand, TATI stabilizes the gut against noxious agents such as indomethacin and stimulates repair after dextran sodium sulphate-induced colitis (Marchbank et al. 2007). In ulcerative colitis, a predisposing state for colorectal cancer, reduced TATI expression has been evident in affected areas (Playford et al. 1995).

9.1.3. EGFR and TATI

Study V showed EGFR immunoexpression to be an independent marker for improved outcome, results differing somewhat from those from previous studies. No association between EGFR expression and survival has emerged in colorectal adenocarcinoma (McKay et al. 2002, Spano et al. 2005) nor in Dukes' C CRC patients (Cunningham et al. 2006). In a study of 149 colon cancer patients, EGFR expression was an independent prognostic marker and associated with poor prognosis, although the EGFR expression was observable only in 35.6% of samples, less than usually reported (Galizia et al. 2006). Resnick et al. (2004) also showed an association between strong EGFR expression and poor prognosis in colon cancer, results differing from ours.

In one study of 87 rectal cancer patients who underwent preoperative radiation therapy, EGFR expression in pre-treatment biopsies, but not in surgical samples, associated with worse prognosis (Giralt et al. 2005). One study by Fernebro et al. (2004) on 269 rectal cancer patients showed no association between EGFR expression and metastasis-free survival. EGFR expression has been studied as a predictive marker for tumour response to neoadjuvant radiotherapy, with an association between positive EGFR and lack of complete pathologic response, but not clinical response (Giralt et al. 2005). Controversially, in a study by Zlobec et al. (2008), pre-treatment expression of EGFR was an indicator of complete pathologic response to preoperative irradiation. Kim et al. (2006) showed that low EGFR expression associates significantly with increased tumour downstaging.

Unlike in Study V, radiotherapy may either raise or lower EGFR expression in rectal cancer; preoperative chemoradiotherapy has reduced immunoexpression of EGFR (Debucquoy et al. 2009, Yasuda et al. 2009). Controversially, some EGFR-negative tumours in one report begin to express EGFR after radiotherapy (Giralt et al. 2005), a finding that may explain these varying results between studies. We did not analyze EGFR expression in pretreatment biopsies, but no statistical difference appeared between EGFR expression of tumours with or without short-course radiotherapy.

In agreement with others' findings (Spano et al. 2005, Rego et al. 2010), in CRC samples, EGFR overexpression was noticeable (Study V). Immunoexpression correlated significantly with tumour grade, being more often positive in high or moderately differentiated tumours, in agreement with others' reported results (McKay et al. 2002). On the other hand, correlation between EGFR expression and poor differentiation also occurs (Rego et al. 2010). As in other studies, no correlation appeared between EGFR expression and histology (Giralt et al. 2005, Molaei et al. 2009). Neither was there any correlation between EGFR immunoexpression and Dukes' stage, in concordance with the study by Giralt et al. (2005), whereas Spano et al. (2005) reported a higher percentage of EGFR overexpression in T3 than in T4 colorectal tumours; and Deng et al. (2009), an association in 94 CRC patients between high EGFR expression and high tumour stage. Moreover, intratumoural heterogeneity in expression occurs (Yang et al. 2012), and due to such variable results, EGFR expression is not recommended as a prognostic tool (Spano and Vignot, 2007).

EGFR-targeted treatments are useful clinically for KRAS wt metastasized colorectal cancer. Interestingly, the EGFR antagonist cetuximab has proved effective even for tumours negative for the EGF receptor (Chung et al. 2005). One proposal is that anti-EGFR treatment should be targeted against metastasized tumours, but the concordance between EGFR immunoexpression in primary tumours and metastases remains unclear and differs between studies (McKay et al. 2002, Bibeau et al. 2006, Deng et al. 2009). Some EGFR-positive tumours do not respond to cetuximab therapy (Saltz et al. 2004); the reason may be KRAS mutations , which associate with poor response to anti-EGFR therapy (Bokemeyer et al. 2009, Chang et al. 2009). A novel method for selecting patients for anti-EGFR therapy is a combination of KRAS-mutation analysis and EGFR gene copy-number assessment (Ålgars et al. 2011).

In Study V, EGFR and TATI were co-expressed in 62.8% of CRC tumours, a combination that was an independent prognostic marker for improved survival. Study III showed TATI to be an independent prognostic factor in CRC. In Study V, concomitant immunoexpression of EGFR and TATI was an independent and an even better prognostic factor for improved survival than was EGFR or TATI alone. In pancreatic adenocarcinomas, serine protease-inhibitor Kazal-1, identical to PSTI and TATI, and EGFR are expressed together, and co-expression stimulates proliferation of pancreatic cancer cells through the EGFR/mitogen-activated protein kinase cascade (Ozaki et al. 2009). Based on the results of the present study it seems that TATI may act differently in CRC.

9.1.4. p53 and Ki-67

Study II showed no association between p53 immunoexpression and survival, in agreement with others' findings (Kressner et al. 1996, Leahy et al. 1996). More recently, in rectal cancer but not in the colon, cytoplasmic p53 expression was associated with improved prognosis (Hilska et al. 2005). Cytoplasmic accumulation of p53 has been associated with poor prognosis in cancers of the distal colon (Bosari et al. 1994, Sun et al. 1996) In Study II, p53 immunoreactivity was more often positive in rectal than in colon tumours, as also reported by Russo et al. (2005).

Mutation of the TP53 gene leads to increased risk of death and to failure to respond to irradiation in rectal cancer (Munro et al. 2005), and overexpression of mutated protein in CRC associates with poor prognosis (Yamaguchi et al. 1992, Manne et al. 1997, Kaklamanis et al. 1998). p53 protein expression has been suggested to act as an independent prognostic factor (Maeda et al. 1997), but contradictory data exist; nor has any association bee observed between TP53 mutation and survival (Scott et al. 1991, Mulder et al. 1995, Hilska et al. 2005).

Study II showed no significant association between Ki-67 expression and survival. Varying results have been reported in CRC, with Ki-67 associating with both poor (Palmqvist et al. 1999) and improved survival (Allegra et al. 2003), or having no prognostic value (Jansson et al. 1997, de Jong et al. 1998, Buglioni et al. 1999). In studies on only rectal or rectosigmoid cancer, conflicting results have also emerged. High Ki-67 associates with improved survival (Hilska et al. 2005, Salminen et al. 2005) but also with poor prognosis (Valera et al. 2005).

9.2. Strengths and limitations of study materials and methods

The patients in this study comprise a consecutive series of CRC patients surgically treated between 1982-1998 at one university hospital. Their overall disease-specific 5-year survival with colon cancer was 57.9%, and for rectal cancer was 54.9%. According to the newest statistics of the Finnish Cancer Registry from 2011, cumulative 5-year survival for colon cancer was 60% for male and 61% for female patients; for rectal and anal cancer, 62% and 65% (Finnish Cancer Registry). Several reasons explain this improved survival over time. Firstly, more lymph nodes are examined, which may lead to stage migration and thereby to a shift in postoperative treatment, since adjuvant treatment is given to patients with lymph-node positivity (Galizia et al. 2009, Qiu et al. 2011, Vather et al. 2011). Secondly, in rectal cancer, the TME technique more widely applied after this study period improves prognosis (Martling et al. 2000). Thirdly, Dukes' B patients with known risk factors more usually receive adjuvant treatment.

The patient series of the study was large and well characterized in regards to clinicopathological and survival data. Only 9 patients were excluded for false diagnosis, 9 for synchronous multiple tumour, and 2 for insufficient tissue. One advantage of this patient series is its long follow-up. It can also be an advantage that only a small proportion of these patients received oncological treatment, which clearly can affect survival. In some patients, detailed data of oncological treatments was lacking because they were given in a different department.

On the other hand, use of old patient data has limitations. TNM classification, the grading system, and surgical techniques all have changed. The staging classification at our department in those years and thereby in our studies was the modified Dukes' stage according to Australian Clinico-Pathological Staging (ACPS) (Davis et al. 1984). Today, more widely used for CRC staging is the UICC TNM classification (Compton et al. 2004). Due to altered preoperative diagnostic methods, operative technique, and pathological analysis, we were unable reliably to apply it retrospectively to our data.

The WHO differentiation grading system has been revised during the time-period of this study (Hamilton et al. 2000). This problem was solved by thorough re-evaluation and re-classification of the differentiation grades according to the newest WHO grading system. The protocol of examination of surgical specimens has changed; nowadays more lymph nodes (a minimum of 12) are identified and examined in surgical specimens. This apparently has led to stage migration and better evaluation of stage, thereby leading to more frequent adjuvant therapies and affecting overall prognosis. In this study, the number of lymph nodes examined was not systematically marked in pathological reports, which constitutes one weakness of this study.

During the study period, differences occurred in surgical technique, particularly for rectal cancer. Some patients underwent the TME technique; the rest had surgical resection with less excision of the

mesorectum. In the TME group, half the patients received preoperative 25Gy radiation; and in total 74 patients were treated by preoperative radiotherapy. Patients undergoing TME were included in a randomized trial. As mentioned, both TME technique and preoperative radiation improve results of rectal cancer treatment (Heald et al. 1998, Martling et al. 2000; Colorectal cancer Collaborative group 2001), and different treatment schema may change the prognosis.

The tissue microarray technique is a suitable method for analyzing large numbers of tissue samples. Our TMA series included, as recommended, three spots from each patient (Jourdan et al. 2003) Because tumours may show heterogenous expression of markers, the risk is to miss the most strongly expressing tumour area. Another disadvantage is difficulty in evaluating the expression in stroma. On the other hand, the results of TMAs including multiple samples taken from histologically representative areas have been in concordance with other biochemical and whole-section analysis (Kononen et al. 1998, Kallioniemi et al. 2001).

9.3. Future prospects

The results of this study confirm that MMP-7, MMP-9, TATI, and the combination of TATI and EGFR may serve as prognostic tools in colorectal cancer. The value of MMP-9 as a prognostic tool should be evaluated in larger prospective studies on stage II tumours to learn more about its usefulness for choosing patients for adjuvant treatment. TATI-EGFR expression may serve as a prognostic tool to decide adjuvant treatment for patients with poor prognosis in colorectal cancer.

10. CONCLUSIONS

- MMP-7 was not an independent prognostic marker in colorectal cancer, but strong immunoexpression associated with poor 5-year survival.
- Neither p53 nor Ki67 was an independent prognostic marker in colorectal cancer.
- TATI was an independent prognostic marker in colorectal cancer, and TATI-positivity associated with improved survival, especially in trypsinogen-1- and trypsinogen-2-positive patients.
- MMP-9 was not an independent prognostic marker in colorectal cancer, but in Dukes' B § patients, MMP-9 immunoexpression associated significantly with improved survival. These results could not however be confirmed in the validation series.
- In an extended multivariate model with ten tumour markers and clinicopathological factors, TATI expression proved an independent prognostic factor alongside age, Dukes' stage, location, and WHO grade. Concomitant tumor expression of EGFR and TATI associated with improved survival in colorectal cancer and was a stronger prognostic factor than was TATI-expression alone.

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